A MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDY OF THE CEPHALOPOD POSTERIOR CHROMATOPHORE LOBE.

by

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For Colin, Mum and Dad.

One thing only I know, and that is that I know nothing.

- Socrates

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CHAPTER SIX.

Intracellular recordings from PCL neurons.

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SUMMARY

1. A unique feature of cephalopods is their skin, which contains thousands of chromatophore organs under direct neural control from the chromatophore lobes of the brain. Control of skin colour changes are described in this thesis in terms of the morphology, electrical activity, and pharmacology, of the Posterior Chromatophore Lobe (PCL) neurons, in the cephalopods *Alloteuthis subulata*, *Loligo vulgaris*, *Eledone cirrhosa*, and *Octopus vulgaris*. The morphology and electrophysiology of the Lateral Basal Lobe (LBL) neurons and the influence of this lobe on PCL neurons are also investigated.

2. The chromatophore lobe neurons are inaccessible in *vivo* and thus a brain slice preparation was developed to allow access to the motoneurons in the PCL. This allowed a study of the morphology, electrical activity, and pharmacology of these neurons to be carried out.

3. An artificial sea water solution was developed to provide a suitable bathing medium for the brain during slicing and recording procedures. The brain slice could be maintained for up to eight hours using this medium, and stable intracellular recordings could be obtained.

4. Over 500 neurons in PCL slices, from 146 animals, were impaled with intracellular recording electrodes and of these, 115 were subsequently filled with the fluorescent dye Lucifer Yellow to reveal their morphologies. It was found that PCL neurons are regularly arranged, with the largest cell somata being on the periphery of the lobe. On the basis of differences in the morphologies and

locations of these cells, they could be divided into four types, referred to in this thesis as cell type I, II, III and IV.

5. The intracellular recordings revealed four different types of spontaneous electrical activity: tonic, irregular bursting, regular bursting, and excitatory postsynaptic potentials (EPSPs). Regular bursting activity was only observed in cell type II. EPSP activity was observed in all the cell types but was most predominant in cell type III.

6. Evidence of both dye and electrical coupling between PCL neurons was obtained. The possible implications of this coupling are discussed.

7. In the light of the existing morphological data, the afferent input from the Lateral Basal Lobe (LBL) was established physiologically by electrical stimulation of the LBL tract connecting with the PCL. The effect on the PCL neurons was excitatory or inhibitory. It is proposed that one LBL cell influences many PCL motoneurons via inhibitory and excitatory synapses. Patterning on the skin could therefore be achieved by control of the frequency of activity displayed in PCL motoneurons, which is regulated by the LBL neurons.

8. Extracellular field potentials revealed the extent and nature of the activity in groups of PCL neurons in response to LBL tract stimulation. These responses were largest in the neuropil area, becoming weaker towards the periphery of the lobe. Field potential responses showed that the synaptic connections between the LBL and PCL neurons occurred at the point where the LBL tract enters the PCL neuropil.

9. Application of acetylcholine (ACh) and carbachol to tissue slices, caused tonically active PCL motoneurons to stop firing, while 5-HT caused an increase in the frequency of tonic activity and the number of EPSPs. The application of antagonist drugs of putative neurotransmitters revealed that L-glutamate, 5-HT and ACh receptors are present on the postsynaptic membrane of PCL cells.

10. These results demonstrate that the brain slice is a useful and versatile preparation for the study of the electrical properties and connections of cells in the cephalopod brain and provide the first physiological clues about how PCL neurons may be controlled as they change the colour of cephalopod skin.

CHAPTER ONE
INTRODUCTION

1.1 Cephalopods

The cephalopods are among the most fascinating of invertebrate animals and in them the molluscan body achieves its most evolved and sophisticated expression. One of their most conspicuous features is their skin, which can rapidly change its colour and often its texture. These changes are under the direct control of the brain and consequently enable the animal to make the fastest colour changes in the animal kingdom. It is this unique neural control of colour change that is the subject of this investigation.

Cephalopods are marine predators which have survived by successfully competing with the teleost and elasmobranch fish in all areas of the sea from shoreline to abyssal depth (Packard, 1972). They include the largest invertebrate animals. Living cephalopods are divided into two subclasses: the Nautiloidea and the Coleoidea (Voss, 1977). With the exception of *Nautilus*, all of the modern cephalopods belong to the single subclass Coleoidea, which contains approximately 650 species. The nautiloids are the more primitive group and the only remaining survivors are a few species of the genus *Nautilus*. Cephalopods have discarded their protective external shells, with the exception of *Nautilus* & *Argonauta* (Lane, 1957), in order to occupy a mobile predatory niche within the sea. To compensate for the loss of protection from the shell, the present day cephalopods rely on speed, learning capabilities, highly developed senses, particularly sight, and a complex skin that changes colour which is used for concealment and communication. In this way the skin has become extremely important in the behaviour of these animals (Holmes, 1940; Boycott, 1953; Packard, 1972; Hanlon, 1982; Boyle, 1985; Hanlon & Messenger, 1988).

The subclass Coleoidea includes the familiar cephalopods belonging to the orders Sepioidea (cuttlefish and sepiolids), Teuthoidea (squids) and Octopoda (octopuses). The octopods have eight arms whereas the sepioids and teuthoids have eight arms plus two longer feeding tentacles; together they are sometimes

referred to as decapods (e.g. Young, 1976). A classification of the extant genera of cephalopods can be found in Voss (1977).

Cephalopods occur in all marine habitats. In deep and midwater regions of the oceans they constitute a considerable proportion of the marine fauna. They are also important in shallow water benthic habitats, ranging from shallow intertidal to sublittoral. Octopus and cuttlefish (e.g. *Sepia*) are particularly successful in coastal neritic habitats which are occupied by fast swimming squids such as *Loligo* and *Alloteuthis*.

1.2 The skin and the chromatophores

The skin of cephalopods is composed of several layers (Fig.1.1) (Packard & Hochberg, 1977; Cloney & Florey, 1968), with the chromatophores lying beneath the epidermis. In the skin of *Loligo plei* (Hanlon, 1982), *Loligo vulgaris* (Williams, 1909) and *Sepia officinalis* (Hanlon & Messenger, 1988), the most superficial chromatophores are yellow; below them are the reds and the browns. Beneath the chromatophore layer is the iridocyte layer. This is comprised of the iridophores, and in some cephalopods, leucophores.

Iridophores are widely distributed in the skin. They consist of multilayered stacks of thin platelets (Mirow, 1972), which produce interference colours when viewed from the appropriate angle (Denton & Land, 1972; Land, 1972; Hanlon & Messenger, 1988). Evidence suggests that cephalopods may actively control some of their iridescence (Cooper & Hanlon, 1986; Hanlon et al, 1990). Leucophores have been reported to occur in *Octopus vulgaris* (Packard & Sanders, 1971; Froesch & Messenger, 1978), and *Sepia officinalis* (Hanlon & Messenger, 1988). They are highly branched cells that function as broad band reflectors, scattering light of all wavelengths; thus appearing white in white light, and yellow in yellow light etc. (Messenger, 1974). Chromatophores are the elements contributing most to colour change because they are most subject to transformation.

In contrast to other animals, where chromatophores are single cells (Bagnara & Hadley, 1973), cephalopod skin chromatophores are organs. Each chromatophore organ consists of an elastic pigment sac surrounded by eight to

twenty radially arranged muscle fibres are inserted (Froesch, 1973; Brocco, 1977) (Fig. 1.2) . These muscle fibres are innervated directly from centres in the brain (Boycott, 1953, 1961; Young 1971). This neural control gives the cephalopods an advantage of being able to produce discrete patterns in the skin and gives them the fastest colour change in the animal kingdom (Packard & Hochberg, 1977).

The colouration of the skin is produced by selective expansion and contraction of chromatophore organs (pigment sacs). When the muscle fibres contract the pigment sac expands, expanding the area of contained pigment; when the muscle relaxes, the elasticity of the pigment sac causes it to retract, and the pigment is reduced to a small spot (Fig. 1.3). Each muscle fibre of the chromatophore organ is innervated by excitatory nerve fibres, that arise directly from cells in the brain (Sereni & Young, 1932). These nerves follow a serpentine pathway along the muscle fibre (Fig. 1.2 & 1.3). This arrangement may permit the muscle fibre to change in length without imposing excessive tension on the nerve. Cloney and Florey (1968) give an extensive and detailed account of the structure and function of cephalopod chromatophores and Florey and Kriebel (1969) give the most comprehensive physiological study of the chromatophore nerves to date, using *Loligo opalescens*. In summary, (Florey & Kriebel, 1969) found that the chromatophore nerves are excitatory only. He also found that the dark chromatophores are multiply innervated and when he increased the frequency and amplitude of the stimulus to the dorsal mantle skin, stepwise expansion of the chromatophores resulted. It has also been demonstrated in isolated pieces of *Octopus* skin that the number of expanded chromatophores can be graded by variations of either the stimulus voltage or frequency and that a single motoneurone innervates at least 100 chromatophores (Dubas & Boyle, 1985). In crustaceans, insects, bony fish, amphibians and reptiles, hormones play an important role in colour change. In cephalopods, however, there is no evidence of hormones being involved in colour change.

The skin of cephalopods is used for camouflage, countershading and display, and there is extensive literature on the importance of skin in the behaviour of cephalopods (Holmes, 1940; Boycott, 1953; Packard & Sanders, 1969, 1971; Packard, 1972; Moynihan, 1975; Hanlon, 1982; Hanlon &

Messenger, 1988). The complexity and number of body patterns within the cephalopods differ according to their different habitats and lifestyles. For example, the shallow water cuttlefish *Sepia* has a remarkable repertoire of body patterns, 34 in total, as most of its life is spent concealing itself on the seabed (Ferguson & Messenger, 1991). Conversely squid which are pelagic have a more limited repertoire of colour patterns. *Loligo forbesii* have been shown to display 17 body patterns (Porteriro, Martins & Hanlon, 1990) and *Alloteuthis* display only 9 recognised patterns.

1.3 The brain and the control of the chromatophores

The brain size of cephalopods approaches that of the lower vertebrates, being the same size as the brain of many fish and reptiles, but smaller than the brains of birds and mammals (Packard, 1972). It is composed of a compact mass divided by the oesophagus into a suboesophageal region, consisting of the lower and intermediate motor centres, and a supraesophageal region containing the higher motor centres (Fig. 1.4). The central part of the brain is enclosed in a hard, cartilaginous cranium. The supraoesophageal region of the brain receives, and integrates inputs from most of the sense organs and is also involved in learning and memory (Boycott & Young, 1950; Young, 1960a, 1960b, 1961a, 1961b; Boycott, 1961; Sanders, 1970). Young (1971, 1976) distinguishes three major regions of the suboesophageal mass in *Octopus* and in *Loligo*: the posterior suboesphageal mass, the middle, and anterior suboesphageal mass. The posterior region is made up of several lobes with various functions. These are: the fin lobes, the posterior chromatophore lobes (PCL), the magnocellular lobes, and palliovisceral lobes (Young, 1976) (Fig. 1.5).

The chromatophores on the mantle skin are innervated by the axons of cells lying in the PCL, but these lobes are believed to be influenced by several of the higher centres located in the supraesophageal mass, including the paired optic and lateral basal lobes (Boycott, 1953, 1961; Boycott & Young, 1950; Young, 1971; Froesch, 1972; Chichery & Chichery, 1976; Novicki et al, 1990) (Fig. 1.6). The available data suggests that the main neural control pathway for the chromatophore system is:

eye → optic lobe → lateral basal lobe→ chromatophore lobes → chromatophores.

Much of the nervous apparatus for the selection of colour patterns is thought to reside in the optic lobes. It has also been shown that extracellular electrical stimulation of the lateral basal lobe, posterior and anterior chromatophore lobes (PCL & ACL) causes the skin chromatophores to expand and stimulation of the optic lobes can produce patterning (Boycott, 1961; Chichery & Chichery, 1976). Because of this experiment and another in which the optic tracts were severed, causing disappearance of pattern on the skin (Andrews et al, 1983), it is probable that the eyes play an important role in selecting body patterns and modifying them in association with events taking place in the animal's visual environment (Boycott, 1961; Young, 1971; Packard & Sanders, 1969; Chichery & Chichery, 1976). It has been assumed that the cells ultimately responsible for each motor program (i.e. each body pattern) reside in the optic lobes (Packard & Sanders, 1969; Young, 1971) but no pattern generating cells have yet been identified. Somehow visual signals from the optic lobe are relayed to the lateral basal lobe, which in turn influences the chromatophore lobes.

Morphological studies have shown that the optic lobes send fibres to the lateral basal lobe (Young, 1971; Saidel, 1982), which in turn send descending tracts to the anterior and posterior chromatophore lobes. These chromatophore lobes contain the final chromatophore motoneurons (Fig. 1.7). The lateral basal lobe is possibly influenced by the statocyst, the median basal lobe and the peduncle lobe, but the major input is derived from the optic lobes (Messenger, 1967; Young, 1971, 1976). The lateral basal lobes can be distinguished externally as slight swellings on the lower posterior face of the supraoesophageal mass on either side of the median basal lobe. The lateral basal lobes form a link between the optic lobes and the chromatophore lobes and are concerned mainly with the control of the chromatophores, although they also influence skin texture (Boycott,

1967; Froesch, 1972; Young, 1971, 1976). In the light of the existing morphological data described above, for the first time this study investigates the lateral basal input onto the PCL and its influences on the chromatophore system.

The appearance of the skin is controlled directly from specialized centres in the brain; the anterior and posterior chromatophore lobes (ACL & PCL). In *Octopus* these lobes contain over half a million neurons (Young, 1971). Classical morphological studies have shown that in *Sepia*, *Loligo* and *Octopus*, the somata of the motoneurons innervating chromatophores are situated in the suboesphageal part of the brain, in the paired posterior and anterior chromatophore lobes. These studies include Cajal silver staining, (Messenger, 1979; Young, 1971, 1974, 1976, 1977, 1979), retrograde transport of HRP (Dubas et al, 1986 a&b), cobalt iontophoresis (Novicki et al, 1990), experimental nerve degeneration in *Octopus vulgaris* (Sereni & Young, 1932), and extracellular stimulation of parts of the brain of the cuttlefish *Sepia officinalis* (Boycott, 1961).

It has been suggested that not all neurons in the posterior chromatophore lobe are chromatophore motoneurons, but that some PCL neurons project to other peripheral structures such as the skin muscles. It has also been suggested that the chromatophore motoneurons are not exclusively restricted to the chromatophore lobes (Dubas et al, 1986b). It was assumed for a long time that the posterior chromatophore lobes innervate the mantle chromatophores exclusively, whereas the anterior chromatophore lobes innervate those of the arms and head (Boycott, 1953, 1961; Young, 1971, 1976). However in *Octopus* it has been found by centripetal cobalt filling of the arms, that filled fibres occur not only in the anterior but also in the posterior chromatophore lobes (Budelmann & Young, 1984). In the squid *Loligo*, however, it seems that PCL motoneurons only run to the mantle (Budelmann & Young, 1984).

Each PCL lies on the dorsal side of the posterior suboesophageal mass, and joins the palliovisceral lobe below (Fig. 1.4). The ACLs are less sharply distinguished from neighbouring regions than the PCLs; they are only apparent as slight swellings of the pedal lobes. Each of these lobes has a separate connection with the lateral basal lobe and there is no evidence that fibres ascend from the chromatophore lobes to the supraoesophageal lobes. The chromatophore lobes on

either side are linked by commissures (Fig. 1.7) and fibres of the lateral basal to the ACL and PCL contribute to this commissure (Young, 1971). The commissure is assumed to co-ordinate the activities of the two sides of the chromatophore lobes. The PCL and ACL on the same side of the animal are joined together by the interchromatophore tract (Fig. 1.7), which may also contain fibres of the lateral basal lobe to the PCL and ACL tract. By these arrangements the patterning over the whole body can be effectively co-ordinated.

The axons of the PCL motoneurons travel to the skin via the pallial nerve, which is a complex nerve carrying at least five different sets of fibres and forming three major nerves: the posterior head retractor nerve which includes three second order giant fibres), the mantle connective, and the fin nerve (Young, 1976). Each chromatophore fibre runs from its origin in the brain straight to the skin through the stellate ganglion. It has been suggested by nerve dengeneration experiments that chromatophore axons do not synapse within the stallate gangilon but travel straight though and exit via the various stellar nerves to terminate on the chromatophore muscles of the skin, mantle and fins (Sereni & Young, 1932; Young, 1973) (Figs. 1.6 & 1.7). It has been demonstrated that a single axon innervates a large number of chromatophores (and within a single chromatophore several muscle fibres) not all clustered together but scattered over a large part of the chromatophore skin (Dubas & Boyle, 1985; Maynard, 1967).

1.4 Neurotransmitters

1.4.1. Chromatophore neurotransmitters

L-glutamate is likely to be a major excitatory transmitter at the neuromuscular junctions on the chromatophore radial nerves (Bone & Howarth 1980; Florey et al 1985; Cornwell & Messenger, 1995). Bone and Howarth (1980) first showed that L-glutamate applied topically caused the chromatophores to expand and this has been confirmed subsequently; non-NMDA type agonists of L-glutamate also cause darkening (Cornwell & Messenger, 1995). More importantly, immunohistochemical staining of the skin with antibodies raised against L-glutamate, shows that this transmitter is endogenous in the radial muscle nerves (Cornwell & Messenger, 1995). Messenger (1991) tested such

glutamate receptor antagonists as gamma-D-glutamylaminomethylsulphonic acid (GAMS) and 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX) on *Alloteuthis* skin and found that they immobilised the chromatophores and subsequently blocked applied L-glutamate. This provides further evidence for the presence of glutamate receptors (of a non-NMDA-type) on the chromatophore radial muscles. They suggested that L-glutamate is therefore endogenous in chromatophore motoneurons.

Serotonin (5-hydroxytryptamine, 5-HT) was shown long ago to antagonize spontaneous tonic contractions of the chromatophores in the skin, and to cause paling on the skin (radial muscle relaxation) when applied topically (Florey, 1966). Similar effects have now been obtained with 5-HT receptor agonists (Cornwell & Messenger, 1995). Immunohistochemical staining has revealed that 5-HT is also endogenous in the radial nerves. They suggested that 5-HT is therefore also endogenous in chromatophore motoneurons.

1.4.2. Brain neurotransmitters

In cephalopods there is a substantial amount of evidence to suggest that the CNS contains biogenic amines and some evidence that these may function as neurotransmitters (Kime & Messenger, 1990).

The presence of dopamine (DA), noradrenaline (NA), and 5-hydroxytryptamine (5-HT) in the brain, has been shown by fluorimetric, enzymatic, thin-layer chromatography and other methods (Juorio, 1971; Juorio & Killick, 1972; Juorio & Barlow, 1973, 1974; Juorio & Molinoff, 1971, 1974). Histochemical studies, which employed the amine specific-induced fluorescence, have also shown the occurrence of DA, NA, and 5-HT in many regions of the brain (Barlow, 1977; Tansey, 1980) (Table 1.2).

Immunohistochemical investigations (Uemura et al, 1987) have provided evidence that 5-HT is widely distributed in the CNS of *Octopus* and is found to be present in some of the PCL and lateral basal lobe cells. It has also been found that the PCL of *Octopus* contains high amounts of NA and that the lateral basal lobes contain DA and NA (Kime & Messenger, 1990). Physiological experiments have demonstrated that there are receptors in the CNS sensitive to DA, NA, and

5-HT (Andrews et al ,1981,1983; Chichery & Chichery, 1985). Tansey (1979) found evidence that ACh may be a possible neurotransmitter in the brain of cephalopods.

We have already mentioned the effects that various neurotransmitters have when applied to the skin (Section 1.4.1. & Table 1.1), but what of their effects on the skin when applied to brain. When ACh is injected into the blood supplying the brain of *Octopus* the chromatophores on the skin retract, causing the skin to pale (Andrews et al, 1983). This effect is partially antagonized by the drug D-tubocurarine. L-glutamate caused an expansion of the chromatophores and darkening of the skin. Andrews et al (1983) also showed that by injecting 5-HT into the cephalic aorta of *Octopus* a mottled pattern was produced, and that this could be antagonized by injection of methylsergide maleate. Brain lesions have been used to localize the sites of action of ACh and 5-HT; these have implied that they act at the level of the suboesophageal lobes to control the chromatophores, but that 5-HT may act at the level of the optic lobe (Andrews et al, 1983).

1.5 Previous work on neural control of behaviour

The evolution of the highly developed and concentrated nervous system seen in the present day cephalopods enables them to exhibit the most complex types of adaptive behaviour to be found in the non-vertebrate world. Consequently the cephalopod gross anatomy and neuronal pathways are well known. Also much has been learnt about their learning and memory functions.

The majority of behavioural studies involving the brain have been carried out on the cuttlefish and octopus, mainly due to their excellent tolerability to surgery, well documented neuroanatomy, and advanced behaviour. These studies have yielded important results, especially about learning and memory (Young, 1960a ;1960; 1961a; 1961b; 1971). This work has been based mainly on discrimination learning, surgical interference with the brain, and electrical stimulation of specific regions in the brain (Boycott ,1961; Young, 1961a, 1971, 1974, 1976, 1977, 1979; Wells, 1962, 1966 a,b,c, 1978; Sanders, 1975; Messenger, 1979; Dubas et al, 1986a; Novicki et al, 1990).

Boycott (1961) provided the first clues as to the functional organisation of the cephalopod brain. He showed that by electrically stimulating the various lobes of the brain of the cuttlefish, *Sepia officinalis*, the lateral basal lobes, anterior chromatophore lobes, and the posterior chromatophore lobes produced expansion of the chromatophores on the skin, while the optic lobe resulted in both expansion and contraction of the chromatophores.

Young (1971) showed in the octopus that the PCL neuropil is well structured with bundles of afferent fibres forming pockets. He suggested that there is some special system of excitation of the cell bodies, other than through their collaterals in the neuropil, and that dendritic branches on the cell body might exist that could provide some clues as to this excitation. Young (1976) produced the most comprehensive and detailed morphological account of the nervous system of *Loligo* to date. He concluded that in the PCL there is no inner layer of small cells, or short-axon cells. He also noted swellings of various sizes and shapes on the dendrites of the PCL. Novicki et al, (1990) used colbalt iontophoresis to establish the brain pathways controlling the chromatophores of the squid. This work confirmed that of Young's (1976).

It was Dubas et al (1986a) who re-examined Boycotts (1961) work on specific functions of the lower motor centres. In keeping with Boycotts (1961) findings, Dubas et al (1986) established that chromatophore motoneurons are located in the posterior chromatophore lobes. They used retrograde transport of horseradish-peroxidase from the chromatophores in the mantle skin of the squid *Lolliguncula brevis* to localize chromatophore motoneurons in the brain and also gave the first direct evidence that chromatophore motoneurons do not synapse in the stellate ganglion. They also stimulated the surface of the PCL and concluded that adjacent motoneurons in the PCL do not necessarily stimulate adjacent chromatophores on the skin. This study, however, was limited to stimulating the surface of the PCL and therefore cells close to the neuropil could not be stimulated. Another limitation of this semi-intake preparation was that no information was obtained on how and where colour on the skin was changed, and recordings were never made.

Much of the neurobiological work carried out on cephalopods has been directed towards fundamental questions of neurophysiology and not directly towards the neural control of behaviour of the animal. For example, the giant axon of squids has provided great advances towards understanding the basic mechanisms of nervous transmission. It was the pioneering work of Hodgkin & Katz (1949) which clarified the idea that sodium was indeed involved in the generation of the action potential and that changes in the external sodium concentration affected the amplitude of the action potential. They concluded that the action potential was the result of a large, transient, increase in the sodium permeability of the membrane.

The wealth of information on the neurobiology of cephalopods discussed above is, however, in contrast with the limited data from electrophysiological recordings from the cephalopod brain. Two reasons may account for this. One is the difficulty in adequately immobilizing cephalopods for stable intracellular brain recordings, and the other is the poor survival rate of the animals when anaesthetized or perfused. A number of anaesthetic agents have been found to be effective (Andrews & Tansey, 1981; Messenger *et al*, 1985), but no satisfactory system has yet been developed for maintenance and control of the level of anaesthetic. Also apart from the giant fibre system, most nerve cells are small, and the central and peripheral ganglia are often firmly ensheathed in connective tissue. Because of these difficulties only three papers (Laverack 1980; Bullock & Budelmann 1991; Miyan & Messenger1995) have been published which describe electrophysiological recordings from cephalopod brains, either in isolated brain preparations or in heavily restrained animals.

Laverack (1980) used a whole brain preparation of the octopus *Eledone cirrhosa* and made impalements of cells within the suboesophageal mass. He obtained resting potentials of between −30 and −60 mV. Spontaneous activity with small spike amplitude (4-10 mV), and bursting cells were recorded. He could not, however, identify where and in which cells he was recording from, and intracellular recordings could only be maintained for a few minutes at maximum

because of movements during perfusion. This work was useful in establishing that the octopus brain does indeed exhibit electrical activity.

Bullock and Budelmann (1991) further investigated the work of Boycott (1961) on an intact unrestrained cephalopod preparation for experiments on brain function. Bullock & Budelmann, however, used recording electrodes rather then stimulating electrodes and compound field potentials and spikes during sensory stimulation of different areas of the brain were observed. Compound potentials were similar to those observed in mammals and other vertebrates, slow waves being one of the similarities. Again as in the Laverack study (1980) the location of the recordings and positioning of the electrodes proved difficult to distinguish. However, the data provided valuable information on the brain activity associated with normal animal activity. Only two other reports deal with compound field potentials in cephalopod brain (Bullock 1984; Bleckmann et al. 1991).

Miyan & Messenger (1995) used a whole brain preparation of octopus which provided the first intracellular recordings from the posterior chromatophore lobe. They penetrated 100 cells and found that they were either silent, displayed spontaneous activity, or displayed regular bursting activity. The membrane resting potential of the cells ranged from −20 to −40 mV and the recorded spike amplitudes were small in all the cells, ranging from −2 to −10 mV. Lucifer Yellow fills confirmed that many cell bodies have dendrites as well as axonal processes with branches, which is unusual for invertebrates. The whole brain preparation used in this paper, however, meant that cells were mainly recorded and dyed from the periphery of the lobe. The inner layer cells were inaccessible and therefore not explored. The influence of the higher centres of the brain was not investigated and the number of cell recordings were low. Movement artefacts due to strong respiration made interpretation of the data obtained very difficult. There was also no correlation between the electrical activity of the cell and its morphology. These two things were treated as separate entities in this paper. This paper showed the first activity of the posterior chromatophore cells but the overall findings were limited.

1.6 Gaps in present knowledge of the neural control of colour change

Despite all the previous work carried out on the neural control of behaviour and despite all the morphological evidence for a connection between the lateral basal lobe and the posterior chromatophore lobe, this has never been confirmed electrically. Therefore we do not understand what influence this critical link between the optic lobe and the chromatophore lobe has on motoneuron activity and thus the effects on the skin.

No investigations have been done on the physiological effects of the possible neurotransmitters mentioned above on individual chromatophore motoneurons, or to establish exactly what effects these neurotransmitters have at the cellular level.

Despite strong and extensive research (Florey, 1966)) there is no evidence for inhibitory innervation of the chromatophore organs on the skin. Which poses the question as to whether an inhibitory mechanism is present in the chromatophore lobes, perhaps at the cellular level. The patterning of the skin on application of 5-HT to the brain and the evidence that it is endogenous in chromatophore motoneurons opens the discussion as to the role of 5-HT within the chromatophore lobe, which still remains unclear.

Despite the extensive and detailed morphological studies on the cephalopod brain (Young, 1971, 1976,1977,1979; Novicki et al, 1990), no detailed study of the morphology of PCL neurons and corresponding electrical activity thoughout the lobe has been attempted. The communication between PCL neurons and the possibility of electrical coupling has also not been investigated.

These gaps in our present knowledge on neural control of colour change can be summarised as a number of questions:

(1) What effect does the higher centre of the brain have on chromatophore motoneurons?.

(2) What neurotransmitters are involved at the chromatophore lobe level ?

(3) What effects do they have on chromatophore motoneuron activity ?.

(4) Is there any inhibitory or excitatory mechanism at present in the chromatophore lobes ?

1.7 Brain slice preparation

In order for the above questions to be answered, the difficulties and limitations of the experiments described in section 1.5, associated with the cephalopod isolated brain and the heavily restrained animal, have had to be overcome by the recently developed cephalopod brain slice technique. This has allowed for stable intracellular recordings to be made from any area of the brain. To date only one paper has been published on a cephalopod brain slice.

Williamson & Budelmann (1991) studied *Octopus* oculomotor neurons, and produced the first intracellular recordings from identified neurons in the cephalopod brain. By using this preparation they could take intracellular recordings from neurons while stimulating a nerve, or record from several nerves while stimulating brain neurons. They concluded that the brain slice preparation is a suitable tool to examine the properties of identified neurons in the cephalopod brain, and their connections with other parts of the brain. Although the preparation used an octopod (*Octopus bimaculoides*), it was thought that slices from decapod species should function just as well.

For many years thin brain slices have been successfully used in many types of research on the physiology of the vertebrate brain.

The technique of recording from brain slices was first developed by Yamamoto and McIlwain (1966) who showed that isolated slices of pre-pyriform cortex of the rat could be maintained *in vitro* and still show activity comparable to that obtained from the intact preparation. By the 1970s the use of the brain slice for metabolic and neurophysiological studies began to attract the attention of many neurophysiologists. The brain tissue used in these studies was, for the most part, mammalian hippocampus, which is still the tissue most widely used for brain slicing because of its unique laminated geometry and the interest in long-term potentiation (LTP). Throughout this period, results obtained *in vitro* were compared with those seen *in vivo* as a means of validating the use of the brain slice technique. In the 1980s brain slices were being applied to many other areas of the brain of mammals, including the hypothalamus, striatum, spinal cord, brainstem, neocortex, cerebellum, and developed into one of the most widely

used in vitro preparations in neuroscience. This is mainly because it has the following advantages:

1) The extracellular environment can be controlled and changes easily made.
2) No anaesthetics are necessary, facilitating the study of synaptic transmission and the properties of neuronal membranes.
3) Groups of neurons and even single neurons can be visualized.
4) Intracellular recordings are readily obtained because there are no disturbances from pulse and respiration: recordings can often be continued for hours, even from small cells.
5) The depth of penetration is small (usually less then 400μm) so that the fine microelectrode tip is less likely to be damaged, compared with *in vivo* preparations in which the recording electrode must pass through a long length of brain before it reaches the target area.
6) Uncontrolled interaction with other brain regions is absent.
7) Finally, an advantage of the brain slice over tissue cultures, is that neuronal circuits may be intact.

1.8 Aims of the investigation

The aim of this investigation has thus been to develop the brain slice technique in order that the unanswered questions outlined in section 1.6 can be solved.

At present, surprisingly little is known of how cephalopods control and regulate colour change. As mentioned above this is mainly due to difficulties involved with immobilizing the animal and gaining access to the brain. Even with the development of a whole brain preparation (Miyan & Messenger 1995) we are still no clearer to answering some fundamental question on the neural control of colour change.

A number of approaches were used to solve the gaps in our knowledge about the neural control of colour change:

1) Development of an artificial sea water (ASW) solution to keep the brain slice and cells in good condition.

2) An examination of the morphology of posterior chromatophore lobe (PCL) motoneurons.

3) An investigation into the electrical activity of PCL cells and dye and electrical coupling between neighbouring PCL cells

4) An examination of the morphology and electrical activity of Lateral basal lobe (LBL) cells. The influence of this lobe on PCL cells was investigated at the individual cell level and from extracellular field potential recordings.

5) A pharmacological investigation into the effects of putative neurotransmitters, agonists and antagonists, on individual PCL cells and on synaptic transmission.

Each of these areas is dealt with as a separate chapter in this thesis.

Two species of squid, *Loligo vulgaris* and *Alloteuthis subulata,* and the octopods *Eledone cirrhosa* and *Octopus vulgaris* available at different times of the year, were employed for the study.

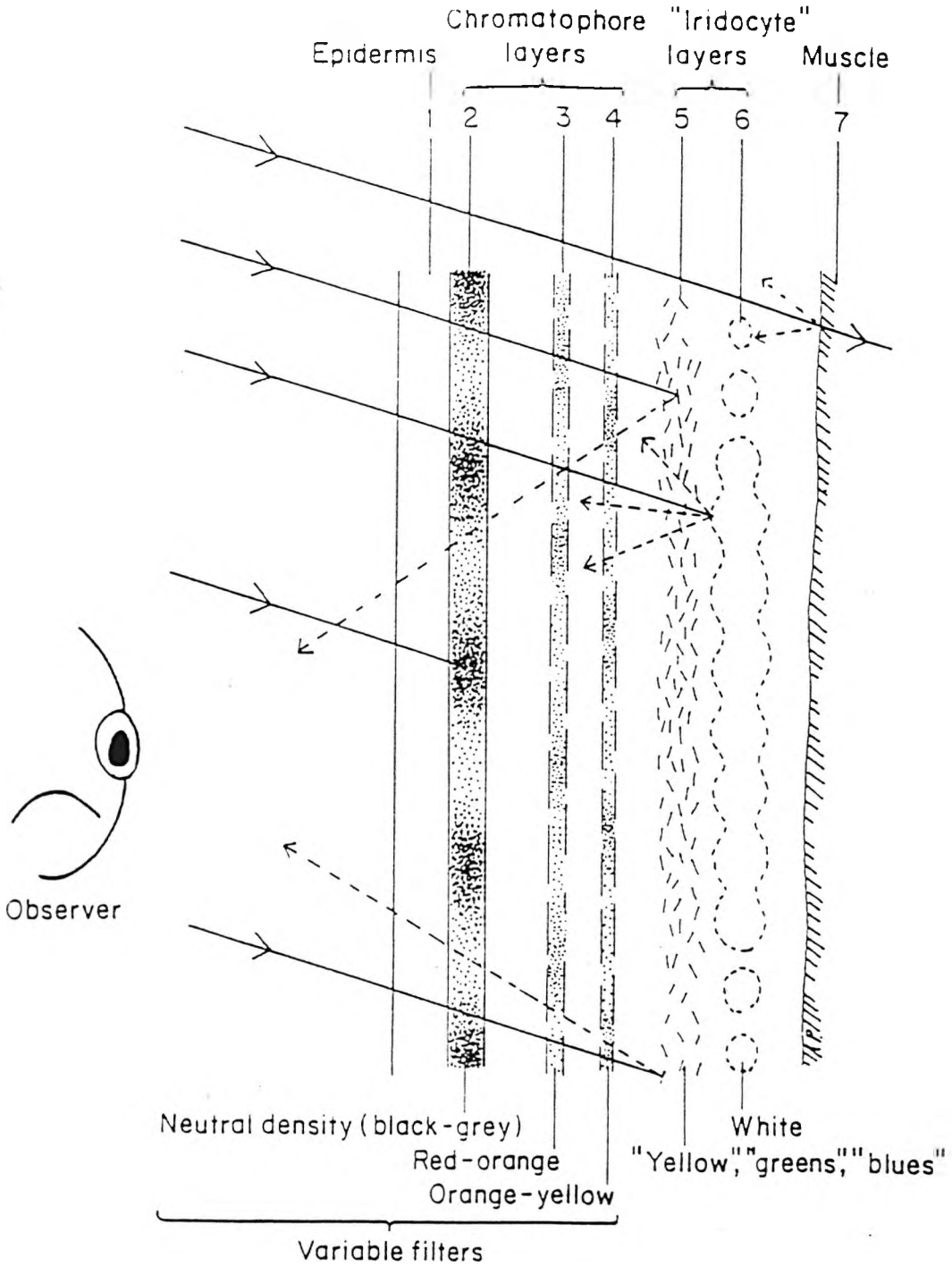


Fig 1.1 Schematic view of the skin of *Octopus* to illustrate its optical properties with respect to an observer. Light successively passes through the refracting layer 1 and the chromatophore layers before being reflected or absorbed by layers 5, 6, and 7. The mirror reflecting layer 5 reflects light in directions that depend on the orientation of the iridocyte platelets composing it. The strongly reflecting white backing layer 6 scatters as well as reflects; it is composed of leucophores. (From Packard & Hochberg, 1977).

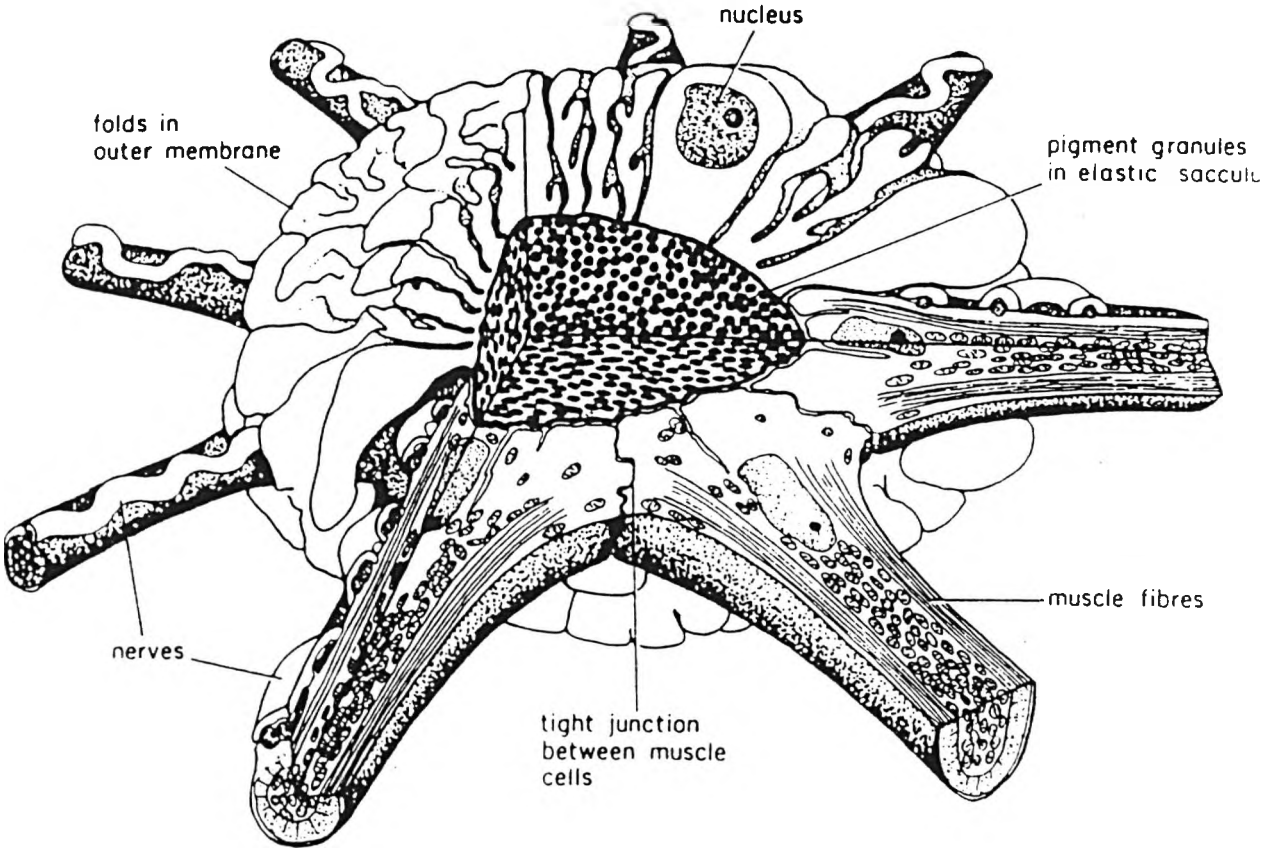
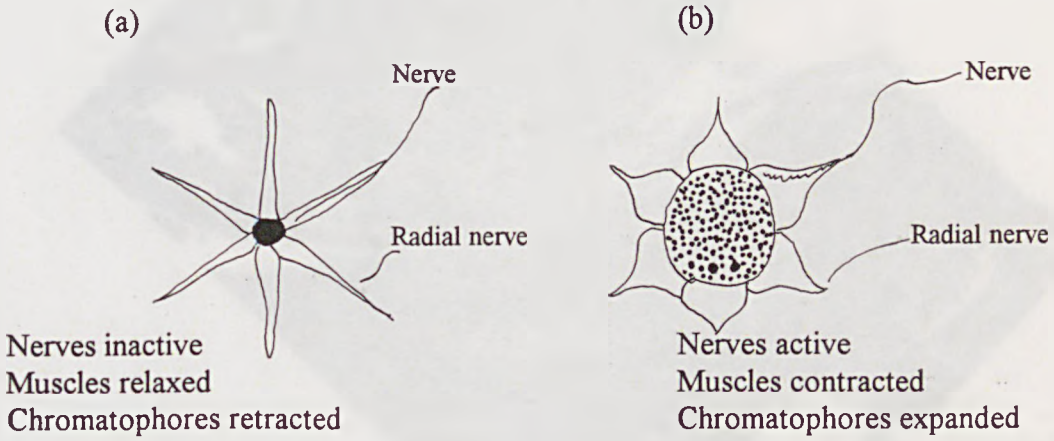


Fig 1.2 Chromatophore organ of the squid *Loligo opalescens*, with the muscles relaxed. In this condition the pigment sac would have a diameter of about 50μm. (From Cloney & Florey, 1968).

(A)



(B)

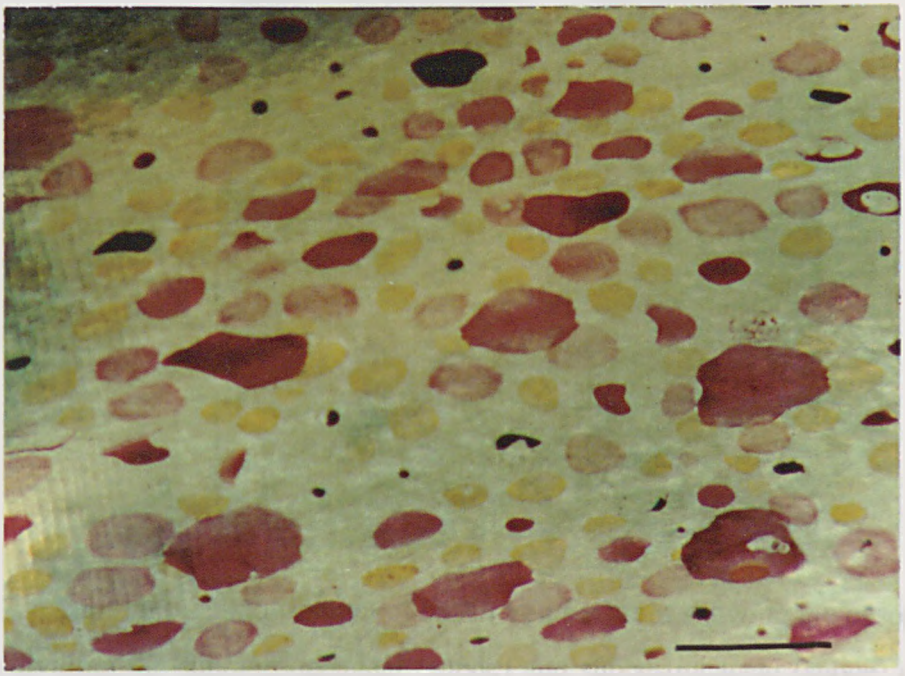


Fig 1.3 (A) Diagram of a squid chromatophore organ retracted (a) and expanded (b). For simplicity a single nerve fibre is shown. Note how the nerve folds when the muscle is contracted (From Cornwall & Messenger, 1995). (B) Photograph of skin from the mantle of *Loligo vulgaris*, showing the different coloured chromatophores at different stages of retraction and expansion. Scale bar: 300μm

Fig 1.4

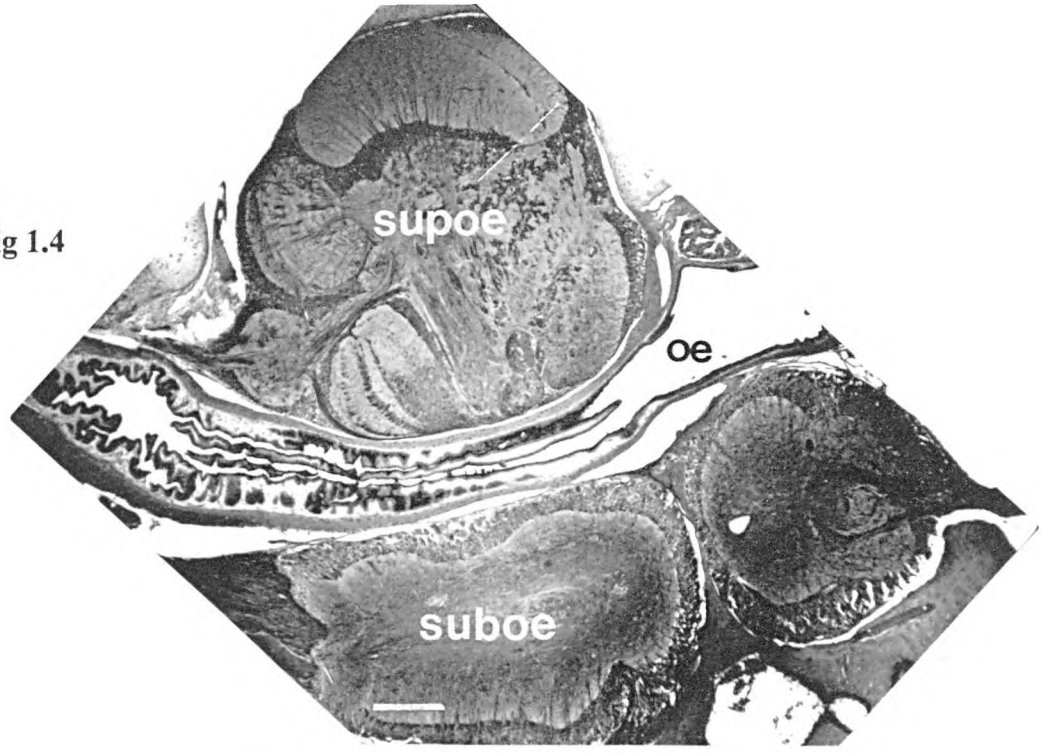
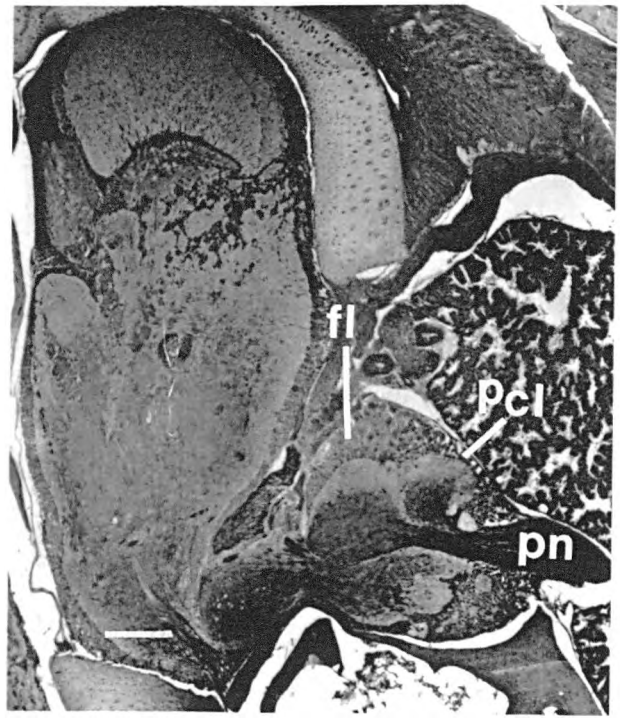


Fig 1.5



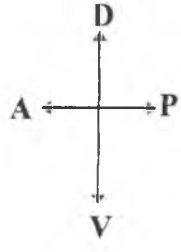


Fig 1.4 A midline sagittal section of an *Alloteuthis* brain showing the oesophagus (oe) dividing the supraoesophageal (supoe) and suboesophageal (suboe) regions of the brain. Caja silver. Scale bar: 500 μm.

Fig 1.5 A lateral sagittal section of an *Alloteuthis* brain showing the posterior region of the brain with the Pallial nerve (pn), Posterior Chromatophore Lobe (pcl) and the Fin Lobe (fl). Caja silver. Scale bar: 500 μm.

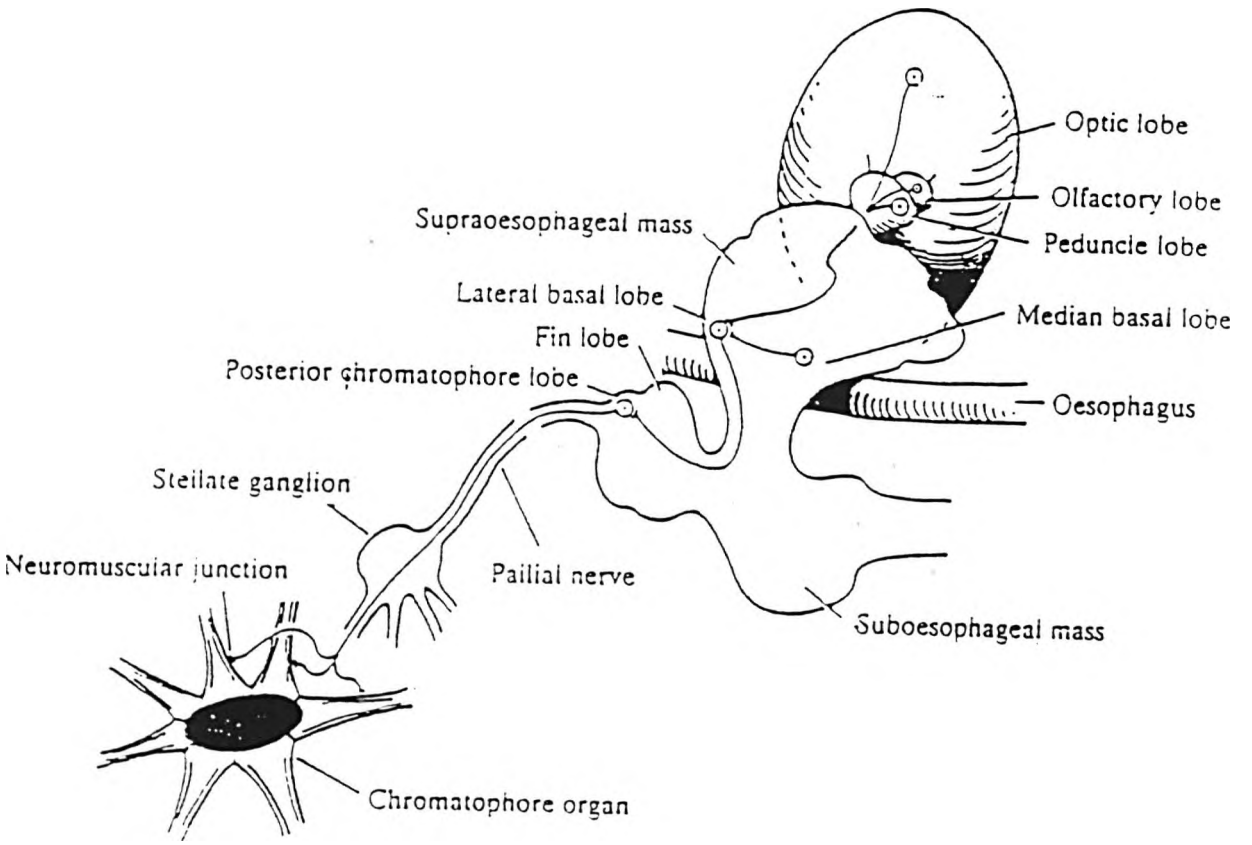


Fig 1.6 Diagram of the main lobes in the cephalopod CNS that control chromatophore patterning. For simplicity only one side of the brain is represented and the anterior chromatophore lobe has been omitted (From Dubas et al, 1986a).

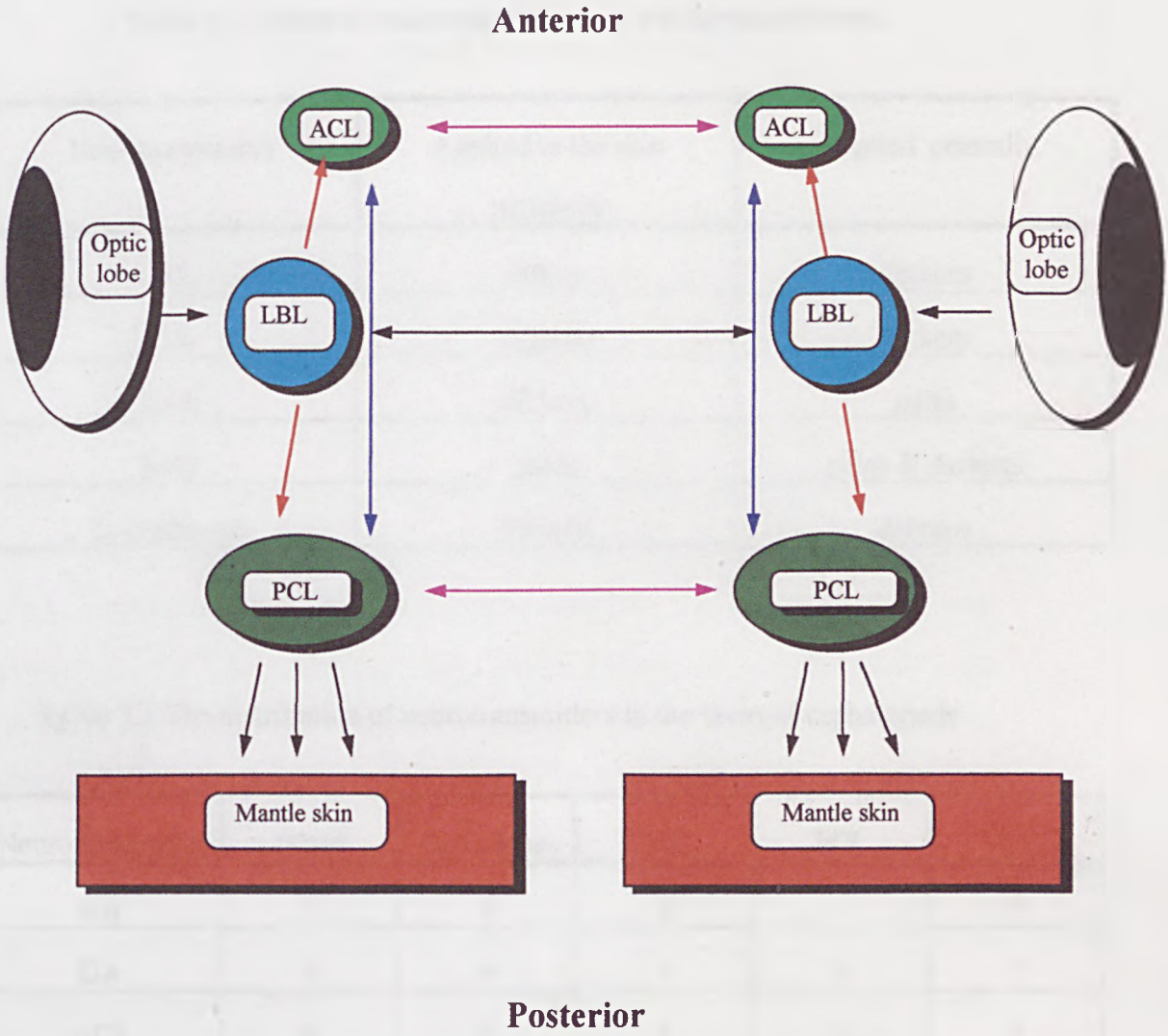


Fig. 1.7 A representative diagram of the neural pathways involved during colour change on the skin. The optic lobes on each side, send fibres to the associated lateral basal lobe (LBL). The lateral basal lobes, in turn, connect to the Posterior and Anterior chromatophore lobes (PCL & ACL) via the lateral basal to the chromatophore lobe tract ⟵ . An interchromatophore lobe connective unites the ACL and PCL of the same side ⟷ The ACL and PCL of either side are linked by a commissure ⟵ . The axons of the PCL motoneurons travel to the mantle skin, while the ACL axons travel to the head and arms.

Table 1.1 Effects of neurotransmitters on the chromatophores

Neurotransmitter	Applied to the skin periphery	Applied centrally
NA	darkens	darkens
DA	darkens	darkens
ACh	darkens	pales
5-HT	pales	pales & darkens
L-glutamate	darkens	darkens

Table 1.2 The distribution of neurotransmitters in the brain of cephalopods

Neurotransmitter	Brain	Optic lobes	LBL	PCL	ACL
NA	√	√	√	-	?
DA	√	√	√	√	?
ACh	√	?	?	?	?
5-HT	√	√	√	√	?
L-glutamate	√	?	?	?	?

CHAPTER TWO
GENERAL METHODS AND MATERIALS

2.1 Animals

Adult specimens of the squids *Alloteuthis subulata* (Lamarck), *Loligo vulgaris* (L.), and *L. forbesii* (L.) and of the octopus *Eledone cirrhosa* (L.) were caught off Plymouth by the crew of the Marine Biological Association Laboratory boats. *Alloteuthis* were maintained in large circular tanks which were supplied with aerated, circulating sea water that was biologically filtered and kept at around 14°C. *Alloteuthis* could survive for up to two weeks, depending on the condition of the animals at capture. *Loligo vulgaris* and *L. forbesii* were kept in a large rectangular tank that was supplied with aerated, circulating sea water filtered through the normal aquarium system and kept at around 18°C. Adult male and female animals could be maintained in this way for several weeks, depending on their condition when caught. They were fed on a diet of fresh or frozen fish.

Most of the experimental work was done at the Plymouth Laboratory of the Marine Biological Association, with a few weeks spent in Naples at the Stazione Zoologica working on *Octopus vulgaris* (Cuvier). *Octopus vulgaris* were caught in the Bay of Naples and kept in individual tanks with circulating sea water. They were fed on a diet of live crabs.

2.1.1. Animal identification

The body of *Alloteuthis subulata* is slender, being up to 210 mm in length, with a maximum mantle length of 140 mm, which tapers posteriorly to a fin point. In males the portion posterior to the fins constitutes one-third to one-half of the total mantle length, in females it is less than one-quarter (Fig. 2.1 (a)). Fins become narrow, rounded, together with a heart shape outline in dorsal view (Hayward & Ryland, 1990).

The body of *Loligo vulgaris* is elongate, solid, fleshy and bluntly rounded posteriorly; up to 750 mm long with a maximum mantle length of about 350 mm.

Dorsal surfaces are a densely pigmented reddish brown, ventral surfaces are lighter. The fins are angular, forming an elongate diamond shape in dorsal view, and comprising about two-thirds of the total body length. The arms are about twice the length of the body, angular in section, with the ventral pair being particularly broad and flat. The median suckers of the middle region are three times the diameter of adjacent marginal suckers. (Fig. 2.1 (b)).

Loligo forbesii is very similar to *L. vulgaris*, reaching 600 mm total length. The two species are distinguished by the tentacle club, on which two longitudinal rows of suckers of the middle region are less than twice that diameter of adjacent suckers (Fig. 2.1 (b)).

Eledone cirrhosa has a smooth or finely tuberculate body, up to 500 mm in total length, with maximum arm spread of 700 mm. The arms are slender, finely tapered distally and curled when at rest, with a single longitudinal row of suckers.

Octopus vulgaris has a distinctly warty body, up to 1000 mm total length, with maximum arm spread of 3000 mm. The arms are thick and stout, bearing two longitudinal rows of suckers (Hayward & Ryland, 1990).

2.1.2. Rationale behind the use of different species.

Experiments were performed on the squids, *Alloteuthis subulata, Loligo vulgaris*, and on the octopods, *Eledone cirrhosa* and *Octopus vulgaris. L. forbesii* was not preferred for experimentation as they were infrequently caught by the fishing boats. Therefore, for consistency *Loligo vulgaris* was used.

Different species were used thoughout the study because of availability problems of certain species during different times of the year, for example: the *Alloteuthis* season is from May to September and the *Loligo* season is from October to March in Plymouth. Therefore, *Alloteuthis* were infrequently caught during the winter and when they were, the condition of the animals was very poor. Therefore the species employed for investigation very much depended on the availability of the animal at that particular time.

Alloteuthis was the preferred animal of the investigation, and the majority of the work is therefore based on *Alloteuthis*. This is mainly because of its small

size and therefore relative ease of dissection, its brain is less complex with fewer but larger PCL cells, and simplified colour patterns. The use of different species of cephalopod added a new dimension to the study in that a comparison could be made between the neural control of colour change in the squid and the octopus, which displays a far more complex skin patterning and behaviour (Packard & Hochberg, 1977; Young, 1961b).

2.2 Brain slice preparation

2.2.1. Dissection

The animal to be used for experimentation was transported from the holding tank to the laboratory where it was killed by decapitation. The funnel was then cut away from the ventral side of the head and the head was pinned, dorsal side up, in a dissection dish filled with sea water pre-chilled to 4°C. The optic lobes were removed by cutting the optic tract using fine dissection scissors. The remains of the hepatopancreas were removed carefully from the back of the head in order to expose the two pallial nerves that exit the posterior suboesophageal brain. The visceral nerves, anterior vena cava, posterior salivary glands, and the oesophagus were removed, leaving the pallial nerves well exposed at the posterior margin of the brain. The surrounding cartilage 'cranium' was left intact and trimmed in order to provide a firm base for securing the brain during slicing. With practice, the time taken for the dissection was substantially reduced until the time from decapitation to preparing the brain for slicing routinely took 3-8 minutes, depending on the size of the animal.

2.2.2. Preparation for slicing

The brain, still within the cartilaginous cranium, was glued with cyanoacrylate 'Supaglue' to a Perspex platform. It was fixed lateral side down, ready to be secured in the slicing chamber. A Sylgard block was glued to the platform behind the brain; this helped to stop the sectioned slice from folding over and being left uncut during slicing. It was found that the critical part was in applying the glue: too much, and it contaminated the brain, not enough and the

brain would not adhere to the block. Good mechanical stability of the tissue was essential for making good slices, as this minimized vibration. It was also essential to get the brain into the ice cold artificial sea water (ASW) as soon as possible; the time taken for glueing and placing the brain in the iced ASW usually took less than one minute. The ASW solution that was used contained (in $mmol^{-1}$): NaCl 429, $MgCl_2$ 50, KCl 8, $CaCl_2$ 10, HEPES 20, glucose 10. All the ASW solutions mentioned below, are of this composition, unless otherwise stated.

2.2.3. Brain slicing

Slices were routinely cut in the sagittal plane, thus providing sections with the pallial nerve, posterior chromatophore lobe, and afferent pathways intact (Fig. 2.2). A standard vibrating microslicer (Campden Instruments) was used to slice the brain tissue. The microslice chamber that was used to contain the slice was pre-chilled in the freezer and filled with ice-cold physiological saline. The Perspex platform on which the brain was secured was fixed to the slice chamber. Keeping the tissue cold throughout the sectioning was particularly important, because it slowed cell activity and minimized damage from anoxia. The "jelly-like" consistency of the brain tissue of cephalopods makes it extremely difficult to slice, the ice-cold saline undoubtedly improved the texture of the tissue for slicing. Slices were cut between 350-450μm thickness with a carbon steel, single edged razor blade. The cutting blade was set to vibrate at the highest available frequency (~12 Hz), thus reducing the movement of the brain during slicing and the forward speed of slicing was manually adjusted so that the tissue was never pushed by the blade. When slicing the hard cartilage surrounding the brain, a slower forward speed was required (~10mm/min), whereas the softer brain tissue was better cut at a faster speed (~ 20mm/min). The slices were either transported by using a plastic disposable pipette with the end cut off; or if the slice was small enough it could be sucked gently into a pipette. If the slice was too big to be pipetted, a fine brush was used to guide the slice onto a broad spatula. Both these transportation techniques limited damage to the slice and enabled the slice to be

deposited into the recovery chamber. The less the slice was handled, the better it survived.

2.2.4. Recovery

After sectioning, each slice was immediately placed in a recovery chamber based on that used by Edwards et al. (1985). The chamber contained oxygenated artificial sea water (ASW) solution at 5°C. The slice remained in the chamber for a recovery period of 30-60 minutes. This time period allowed the slice to recover from abnormal activity caused by any mechanical damage resulting from the slicing procedure. During this time the temperature of the saline in the recovery chamber gradually increased to match that in the recording chamber (12-17°C). In order to ensure sufficient oxygenation and saline circulation during the recovery, the slices were aerated directly from below in the chamber. Up to 10 slices at a time could be held using this chamber.

2.2.5. Preparation of the slice for recording.

After the recovery period, a slice was selected for recording if it conformed to the following criteria:

1) It had to contain the posterior chromatophore lobe (PCL), the lateral basal tract and the pallial nerve.
2) The edges of the lobes had to be well defined with no dead (opaque) cells appearing at the slice surface.

The condition of the slice was found to be optimal for the first 3 or 4 hours after slicing; after this period dead cells sometimes appeared on the surface of the slice. Nevertheless, even under these conditions, many healthy cells remained from which stable recordings could be made 6-8 hours after slicing. The recording chamber was perfused continuously with oxygenated ASW solution kept at a constant 12°C-14°C. The chamber was of the submerged type, where the slice lies on a fine nylon grid so that it is exposed to the perfusate on both sides. In this way diffusion of oxygen was achieved from both sides of the slice. For recording, the slice was fixed to the nylon grid by a fine platinum wire

laid across it. If the pallial nerve and lateral basal tract were present and clearly visible, a bipolar stainless steel stimulating electrode (Rhodes Medical Instruments, USA) was placed on the pallial nerve, and a monopolar electrode on the lateral basal tract.

2.3 Artificial sea water (ASW) solutions

To provide a medium for keeping the brain slice during the experiments a solution was developed which was named an artificial sea water (ASW) solution. Four different solutions were tested:

1) Brain jelly ASW
2) Hodgkin ASW
3) Tissue culture ASW
4) Williamson & Budelmann ASW

2.3.1. Protocol for testing the different ASW solutions.

The viability of the different ASW solutions was tested, during the early stages of this thesis, with respect to:

1) Their effect on field potentials in the optic lobe slice and brain slice.
2) Membrane resting potentials (MRP) of the PCL cells.
3) Their effect on EPSP activity displayed by the PCL cells.

The criterion for a ASW solution beginning good was that:

1) It increased brain slice survival time.
2) It reduced the appearance of dead cells or swelling of the slice.
3) It improved the resting potentials of the cell.
4) It improved spontaneous activity and EPSPs.
5) After application of Fluorescien-di-acetate (which marks healthy cells green) there was an abundance of green stained cells.

Field potential recordings in the optic lobe slice were used in the early days of the investigation to test the viability of ASW solution because;

1) It was easy to obtain recordings.
2) Field potentials can be held for long periods of time during perfusion experiments unlike intracellular recordings.

3) They are a convenient way of recording the activity of groups of neurons.

Field potentials were generated by stimulating the optic nerve, and recorded with extracellular electrodes which were held in one location during perfusion of the ASW solutions. The slice was kept in the brain jelly ASW and purfused with Hodgkin ASW and the effect on the field potential was monitored.

As the brain slicing technique improved membrane resting potential (MRP) recordings could be obtained from PCL cells. MRP was monitored by the negative drop in the oscilloscope trace, which is the potential difference between the outside and inside of the cell. MRPs were used as an indication of the overall health of the slice and individual cells. Generally speaking if the cell was damaged in some way either by mechanical damage or from physiological damage, possibly caused by the ASW solution, then there would be leakage from the cell which would effect the balance of charges, resulting in a low potential difference across the cell and a small MRP. MRPs were compared in brain slices kept in the brain jelly ASW and the tissue culture ASW.

It was important for this investigation to have a slice which was kept in a solution that was advantageous to the spontaneous activity of the cells. It would be useless to have a solution which produced good MRP but no activity. Therefore it was decided to investigate the effects of the preferred tissue culture ASW on spontaneous activity mainly in the form of cells displaying spontaneous signs of excitatory postsynaptic potentials (EPSPs). The tissue culture ASW was modified to improve the amplitude and the number of spontaneous EPSP events as well as evoked EPSPs of the cell.

2.3.2. Brain jelly ASW

The rational behind developing a brain jelly solution was to mimic the surrounding environment of the brain. The fluid surrounding the brain has a jelly-like consistency, hence the name ''brain jelly''.

The jelly surrounding the brains of 10 squids (*Loligo vulgaris)* was collected. This was achieved by firstly decapitating the animal and then slicing the top of the cranium to expose the brain. To prevent contamination of the brain fluid, sea water was not used during dissection. Once the brain was exposed, a

fine pipette was used to gentle suck the jelly up. Because the jelly was too thick to be completely sucked up it had to be cut away from the brain. The operation of removing the jelly was not easy due to its consistency. The jelly was then placed into a sterilizsed vile for analysis. A sample size of 10 animals was required to supply enough material for analysis. Unfortunately due to the time taken for this operation and the possibility of damage to the brain, after collection of the brain jelly, the brain was disposed of.

Analysis of the brain jelly was done by using flame atomic spectophotometry, using the method of Bryan et al (1985). The ionic composition of the jelly for Na^+, K^+, Mg^{2+}, and Ca^{2+} was obtained; in $mmol^{-1}$: Na^+ 296, K^+ 13.4, Mg^{2+} 38.7, Ca^{2+} 8.4.

A solution was then made based on the brain jelly analysis which contained; in $mmol^{-1}$: NaCl 300, KCl 14.0, $MgCl_2$ 39.0, $CaCl_2$ 9.0, HEPES 10, Glucose 20. This solution was made up by dissolving into 1000 ml of distilled water; 17.53g NaCl, 1.04g KCl, 7.93g $MgCl_2$, 1.32g $CaCl_2$, 2.38g HEPES, 3.60g Glucose. The equation used thoughout the thesis for the conversion of $mmol^{-1}$ into grams was as follows:

Grams = Molecular weight of the ion (MW) × $mmol^{-1}$ of the ion ÷1000 ml

The pH of the solution was adjusted by adding small amounts of NaOH to pH 7.5 because the plasma surrounding the brain has a similar pH (Abbott et al, 1985). The osmolarity of the final solutions was measured using a vapour pressure omometer (Wescor 5000) and balanced to approximately to 1000 $mOsm\text{-}kg^{-1}$ by adding sucrose, as squid tissue fluids have osmolarities similar to sea water (Skoukimas et al, 1977).

2.3.3. Hodgkin ASW

It was decided to compare the newly developed brain jelly ASW with a well known ASW solution used by Hodgkin & Katz (1949), which had proven successful for experiments on the squid axon. The recipe for the ionic

composition was taken from the same paper and composed of in $mmol^{-1}$: NaCl 470, KCl 10, $MgCl_2$ 53, $CaCl_2$ 11, HEPES 20.

The viability of these two solutions was compared by monitoring the change in the amplitude of field potentials, produced in groups of optic lobe neurons. The optic nerve was stimulated when the Hodgkin ASW solution was purfused onto the slice, and when it was kept in the brain jelly solution.

2.3.4. Tissue culture ASW

The resting membrane potentials of PCL neurons were improved by using a ASW solution based on a formula used for squid tissue culture (Rice et al 1990). This was reported to produce improved resting membrane potentials in squid neurons. The composition of the ASW used in this thesis was that used by these workers: in $mmol^{-1}$ NaCl 430, KCl 10, $MgCl_2$ 50, $CaCl_2$ 10, HEPES 20, Glucose 10. The pH was adjusted to 7.7 and the osmolarity to 800 milliosmoles.

This viability of this solution was compared to that of the brain jelly solution by monitoring resting membrane potentials of PCL cells under both solutions. A slice kept in brain jelly solution was purfused with the tissue culture ASW solution and the resulting changes in the resting potentials of the cells monitored. Resting potentials improved in the tissue culture medium and this solution was preferred to the brain jelly ASW.

2.3.5. Williamson & Budelmann ASW

It was decided as a final comparison, to use an ASW used by Williamson & Budelmann (1991) for work on the cephalopod brain slice. The composition of this ASW was as follows: in $mmol^{-1}$ NaCl 470, KCl 10, $MgCl_2$ 55, $CaCl_2$ 11, Glucose 10, and buffered to 7.6 pH with MOPS. This solution however had no advantage over the tissue culture ASW solution.

2.3.6. Final ASW used thoughout the thesis

Based on the criterion mentioned in section 2.3.1. for the preferred ASW solution, a modified version of the solution used for squid neuron tissue culture was used in all the following experiments. The modification involved a reduction

in KCl from 10mmol^{-1} to 8mmol^{-1} as this increased the number of EPSP events in the PCL cells. With this solution the brain slice remained viable for up to eight hours.

A dye used to label large numbers of cells rather then single cells, Fluorescein diacetate (FDA), was used in this study to identify healthy cells in the PCL and to compare the viabilities of the different ASW solutions. Fluorescein liberated inside cells will only cross membranes of damaged cells, rendering intact healthy cells fluorescent green at FITC wavelength. By incubating a PCL slice kept in 3% FDA, in a modified version of the tissue culture ASW solution, the abundance of green stained cells was revealed, more so then in any other ASW solution. Therefore the solution that was used though-out the rest of the experiments was composed of in mmol^{-1} NaCl 430, KCl 8, $MgCl_2$ 50, $CaCl_2$ 10, HEPES 20, glucose 10. pH 7.6. with an osmolarity of 969 milliosmoles.

2.4 Electrophysiology

2.4.1. Intracellular recordings

Intracellular microelectrodes were used in this study for:

1) Intracellular recordings from posterior chromatophore (PCL) cells and lateral basal lobe (LBL) cells to investigate their membrane properties, electrical activity and coupling.
2) Current injection to evoke electrical activity.
3) Dye injection, following electrical recordings, by iontophoresis into PCL and LBL cells.
4) Recording the effects of applied drugs to PCL cells.

The methods associated with numbers 3 and 4 are dealt with in more detail in sections 2.5.2 and 2.7 respectively. A general account of how intracellular recordings were obtained follows.

Intracellular recordings were obtained by using glass microelectrodes (capillary tubing 150F-15 225 Clark Electromedical, England) filled with 3M KCl, heated and stretched on a vertical puller giving a resistance of 30-40 MΩ. It was found that a hydraulic microdrive (Narashige MO-15N) was essential for

successful penetrations. An impalement was made by slowly moving the microelectrode over a visually selected cell and area until the trace on the oscilloscope became unstable and showed sings of resistance. Successful penetrations were then best achieved with brief capacity overcompensation, which pushed the electrode into the cell. Another method employed, with practice, and which was equally successful was to gently but firmly tap the end of the micromanlipulator so that the electrode "jumped" into the cell.

The criteria for interpretation of both electrophysiological and morphological data were that a single penetration occurred when no sudden changes in membrane potentials were measured during a recording and dye-filling experiment. Intracellular recordings could be held in a single cell for up to 35 min under these conditions.

Passive electrical properties of the cell, such as the resting potential (V_m) and input resistance (R_{in}), were routinely measured 2-5 minutes after penetration to allow the resting membrane potential of the cell to settle. V_m was measured by recording the voltage drop after penetration of the electrode. R_{in} was measured by delivering hyperpolarising pulses of different sizes to the cell and calculating the slope of the current/voltage regression line. Before and after cell penetration the bridge was balanced in order to obtain reliable readings for the cell input resistance.

Spiking activity was induced by the injection of pulsed depolarising current through the microelectrode. Spike amplitude was measured from the baseline to the peak. Spontaneous depolarisation's, some producing spikes, were identified as excitatory postsynaptic potentials (EPSPs) and a single spontaneous event was defined as a depolarisation which was larger than 2mV: anything smaller was taken to be noise. EPSP amplitude was measured from the baseline to the peak. The peaks of the large EPSPs that triggered action potentials, were measured at the point where the action potential was triggered. In order to evoke EPSP activity, the lateral basal lobe tract was stimulated. An ASW solution containing Low Ca^{2+} and high $MgCl_2$ (in $mmol^{-1}$ NaCl 430, KCl 8, $MgCl_2$ 120,

$CaCl_2$, HEPES 20) was used to block, and hence identify, evoked and spontaneous EPSP activity.

The routine experimental protocol was to obtain an intracellular recording from a cell soma in the posterior chromatophore lobe, observe any spontaneous activity, and then stimulate the pallial nerve to evoke an antidromic action potential and hence identify the cell as a chromatophore motoneuron (Section 2.5.1.). The lateral basal tract was then stimulated to identify any input from the lateral basal lobe to that neuron. Finally the cell was morphologically identified by dye filling using iontophoresis. Methods of cell identification are dealt with later in section 2.5).

To investigate cell-cell communication both electrical and dye coupling were employed. Two recording electrodes were placed above the PCL slice and a cell was located. One electrode had been previously filled with a 3% solution of Lucifer Yellow-CH (Sigma), the microelectrode was then backfilled with 3M solution of lithium chloride (KCl could not be used to fill the electrode because it forms an insoluble precipitate with Lucifer Yellow). One microelelctrode was advanced towards the slice and penetrated an identified chromatophore motoneuron. The other electrode was positioned one cell away from the original cell, and carefully advanced into it. LY was injected into one cell by iontophoresis, short pulses of alternating polarity were used to fill the cell. Dye was injected for 5 minutes.

2.4.2. Extracellular recordings

Extracellular recordings were used in this study for:

1) Monitoring optic lobe cell activity, generated by optic nerve stimulation, to test the effectiveness of various artificial sea water (ASW) solutions on cell activity.
2) Recording the activity of groups of PCL cells during pallial nerve stimulation in order to trace the influence of the nerve and the areas of high activity within the PCL.

3) Investigating the influence of the lateral basal lobe input on the activity of groups of PCL neurons though-out the PCL.
4) Observing changes in the activity of groups of PCL cells during drug application.

The methods associated with the different experiments mentioned above have either been dealt with already or will be addressed in more detail. A general account of how extracellular recordings were obtained follows.

Extracellular recordings were obtained from posterior chromatophore neurons using glass microelectrodes filled with 0.5M NaCl and pulled on a vertical puller to give a resistance of 2-5 MΩ. The recording electrodes were placed on the slice, and advanced through it until a maximum field was located. The stimulus strength was adjusted in order to evoke a field potential but was kept to a minimum in order to preserve the condition of the nerves and tracts. The recording electrode was then moved and reinserted into the slice at various positions in order to trace either the extent of the field generated by stimulation of the efferent, pallial nerve fibres, or the afferent fibres from the lateral basal lobe.

Individual field potentials were amplified with conventional preamplifiers and displayed on an oscilloscope. The signal was then sent to a Cambridge Electronic Design (CED) 1401 controlled by the CED 16 channel signal averager programme and PC 486 computer. Multiple traces of one field potential were collected on the computer so that an average of the traces could be made.

2.5 Identification of chromatophore motoneurons

During intracellular recordings one of the requirements was to establish the identity of the chromatophore cell. Several methods were employed to do this: 1) Electrical stimulation of the pallial nerve 2) Intracellular dye injection 3) Retrograde dye filling. A description of the techniques used to identify chromatophore motoneurons follows.

2.5.1. Electrical stimulation

The brain was always sliced to leave intact the pallial nerve (PN). A bipolar stimulating electrode was then placed on top of the pallial nerve, which

served both to secure the slice and provide a method by which associated responses in PCL cells could be observed. A PCL cell was penetration and the membrane potential was allowed to stabilise for a few minutes. The Stimulus to the pallial nerve was gradually increased until stimulus threshold was reached and an all or nothing antidromic action potential was seen. This potential was identified as an antidromic action potential when increasing the stimulus to the PN induced no increase in the amplitude of the response. The response was therefore not an EPSP.

Extracellular field potentials were used to identify areas in the PCL associated with chromatophore motoneuron activity. Field potentials (FPs) were obtained as described in section 2.4.2., and generated by stimulation of the pallial nerve in the same manner as for the intracellular investigation of antidromic action potentials in individual PCL cells. FPs were sampled though-out the slice and recorded on the computer system. A drawing was made of the PCL slice and the position of the recorded FP was marked on the drawing. This was later matched to the morphology of the PN fibres, obtained by retrograde dye transport in the PN of the same slice (2.5.3.).

2.5.2. Intracellular dye injection

After an antidromic response was observed, or not, as this case may be, and sufficient intracellular recordings had been taken, the cell was intracellularly dyed filled using iontophoresis. This enabled the cell morphology and especially the axon to be traced. The electrical identification of a PCL motoneuron could therefore be backed up by the morphology result. The cell axon could be traced leaving the PN and making its way to the skin therefore identifying the cell as a possible chromatophore motoneuron. If no response was observed in the cell by stimulation of the PN, intracellular recordings were still recorded and the cell was later intracellularlly dyed filled and the axon could sometimes be seen leaving the PN, indicating that the cell could be a chromatophore motoneuron.

Iontophoresis was the method used thoughout this thesis to introduce compounds into individual PCL and LBL cells to identify them following

electrophysiological recordings. This method was also used to investigate dye coupling among PCL cells, as well to identify chromatophore motoneurons.

Iontophoresis involves the injection of a substance from a microelectrode by the application of a current. The microelectrodes used for intracellular dye injection were the same as those used for intracellular recordings (section 2.4.1.). However the pipette was filled with KCl and Lucifer Yellow-CH (Sigma). A 3% solution of Lucifer Yellow was introduced into the back of the pipette, by bringing it into contact with a drop of the solution, until it was visible at the tip. The microelelctrode was then backfilled, using a syringe that had been heated and stretched to a fine point, with 3M lithium chloride (LiCl). KCl could not be used to backfill the electrode as it forms an insoluble precipitate with Lucifer Yellow. These electrodes had resistances of between 100-200MΩ. However, the microelectrodes used to study LBL cells had higher resistances (200-250MΩ) due to the smaller size of the cells.

To ensure that LY had no effect on the physiology of the cell, the dye was injected into PCL and LBL cells following electrophysiogical recordings. LY was injected into cells by short pulse of hyperpolarising current (1-5nA) which helped to prevent the electrode from blocking. Dye filling was considered to be complete after 10-20 minutes which was sufficient to fill the fine processes of the cell. During dye filling the resting membrane potential of the cell was monitored at intervals, to check the condition of the cell. Blocking of the electrode seemed to be the main cause of dye failure. To combat this a number of procedures were established

1) A new electrode was used for each dye fill.
2) Small currents for long periods were usually more successful than large currents for shorter times.
3) Pulsed, rather than continuous current was used.
4) Electrode resistance low enough to allow good dye loading but high enough to limit the damage to the cell during penetration.

A blocked electrode could sometimes be rectified by reversing the polarity of the current for a short time.

At the end of a recording session, the slice was fixed overnight in 2.5% paraformaldehyde in 0.1M Na phosphate buffer, pH 7.4. The fixed slice was washed in buffer then dehydrated through a series of graded alcohols (50%, 70%, 90% for 15 minutes each, and 100% for 30 minutes). The slice was cleared in 100% methyl salicylate and then placed within a ring glued onto a specially made slide: the ring was filled with methyl salicylate and cover-slipped. This provided a means of storing the cleared slice for further reference and observation under the microscope. The slice was viewed shortly after dye filling so that post-fixation migration of LY was limited (Stewart, 1978). Slices were viewed using an excitation filter of 350-440nm and emission filter of 540nm.

Cells were photographed under an epifluorescent Nikon Optiphot microscope using 35mm 100 & 400 asa colour film, 400 asa transparency film, and 400 asa black & white film. The colour film was processed commercially using the C41 process.

A PCL motoneuron filled with LY by iontophoresis also had its morphology investigated by a series of images taken on a scanning confocal microscope. The slice was fixed and then cleared, after dye injection, in the same way described above, and transported to a Phoibos 1000 (Sarastro) microscope and viewed at an excitation of 488nM. The image of the cell was scanned at 10 μm sections and each section was photographed directly from the computer screen. The photographed images, taken at 10 μm intervals, were then stacked together by a computer programme, to reveal the cell in its entirety.

Cascade Blue a relatively new dye available from Molecular Probes which shares many of the properties of Lucifer Yellow (Mobb et al) was used in only a few occasions. PCL cells were injected with Cascade Blue and were excited by near UV light at an excitation of 375-400nm and emission filter of 410nm.

2.5.3. Retrograde transport of dye.

This is a convenient method for loading cells in large numbers, rather than singly, and also for dye filling small cells such as LBL cells which are not easily filled by iontophoresis. This method was used to dye the whole pallial nerve (PN) and trace axons of the chromatophore motoneurons back to their cell bodies. This is a useful technique to;

1) Trace the morphology of the PN and where it is running thoughout the slice, therefore being useful for extracellular recordings.
2) Ascertain whether axons are still intact after the slicing procedure.

Retrograde labelling was also used to trace LBL fibers thought the PCL slice for anatomical investigation, and to match the morphology of the PN or the LBL dyed fibers to the position and shape of the evoked FPs.

The method of retrograde labelling of neurons is widely used for tracing anatomical pathways and is covered, for example, in Helmer & Robards (1981). Two methods were used for retrograde labelling; 1) Back-filling of the pallial nerve using LY; 2) placement of a dye crystal in the LBL tract used in DiI application (section 2.6.2)

The back-filling technique was especially useful for pallial nerve identification as this nerve is not a surface lying tract and is reasonably large for handling. This technique involved constructing a small water-tight well of Vaseline in which the dye was placed. The cut end of the nerve was then pinned to the Sylgard dish inside the well containing the dye. The preparation was left overnight at $4^{O}C$ then fixed in 2.5% paraformaldehyde in 0.1M Na phosphate buffer- pH 7.4, dehydrated in 50, 70, 90, and 100% alcohol solutions, and cleared in methyl salicylate. The dye enters the neurons and is transported back to the cell body. The degree to which the pathway was traced depended on the time allowed for axonal transport. DiI was more useful for tracing longer distances as it could be used in fixed tissue.

2.6. Principles and practice behind the use of dyes.

Cells were morphologically identified in this thesis by using various methods for introducing compounds; 1) Iontophoresis (Section 2.5.2.) 2) Retrograde transport (Section 2.5.3.) and 3) Membrane soluble derivatives of fluorescein diacetate (Section 2.3.6.). According to their suitability, three different dyes have been used to dye either individual or groups of cells; 1) Lucifer Yellow 2) Carbocyanine dyes (DiI & DiO) and 3) Fluorescein diacetate (Section 2.3.6.).

2.6.1 Lucifer Yellow

Dyes used in this thesis for labelling cells for subsequent identification and overall cell architecture were used because of their following general properties;

1) They remained in the cell, either because the molecules are too large to move across the cell membrane and though gap junctions or because they are strongly bound by the cytoplasm.
2) They were not toxic, although most of the dyed-filled preparations were processed immediately after filling and this requirement was relaxed.
3) They were stable and did not break down to give products with different properties.
4) They withstood histological processing.

Two classes of compounds have been used in this thesis for the purpose of cell identification, Lucifer Yellow, DiI and DiO. Lucifer Yellow has been used in this study to;

1) Study individual PCL and LBL cell morphologies, following electrophysiological recordings, by iontophoresis (Section 2.5.2.)
2) Study dye coupling by iontophoresis
3) Study the pathway of the pallial nerve though the PCL slice by retrograde dye transport (Section 2.5.3).

Lucifer Yellow-CH (MW 457) is a popular fluorescent compound for determining overall cellular architecture. LY withstands fixation well but as with

all dyes some fluorescence intensity is lost. Some of the advantages of LY include;

1) It is easily injected into cells by Iontophorsis.
2) It is an intensely fluorescent dye and can be seen in living cells with appropriate illumination.
3) Does not break down.

However it is far from ideal for cell identification exclusively. LY has a low MW of 457 so it can pass between cells through gap junctions (Stewart, 1981) which makes it ideal for examining dye coupling.

Dye coupling occurs by the exchange of small molecules from on cell to another through the gap junction. The size and charge of the injected molecules is therefore of importance, because this will determine whether the molecule moves from one cell to the next. LY has a low molecular weight of 457 so that it can pass between cells easily and rapidly (Stewart, 1981), it is also easily injected into the cell by iontophoresis, it is also intensely fluorescent and therefore a highly viable dye.

2.6.2 The carbocyanine dyes (DiI & DiO)

Octadecyl(C18)-indocarbocyanine (DiI) and oxycarbocyanne (DiO) (molecular weights 943 and 882) (Molecular Probes D3886, Oregon) are highly fluorescent lipophilic compounds. They dissolve in, and diffuse through, the lipids of the plasma membrane. DiI has been used in this study to investigate the LBL tract by retrograde labelling in fixed tissue, and DiO has been used for the identification of individual LBL cells by placing flakes of the dye on the cell somata. The main differences between the DiI and DiO are;

1) DiI has a higher solubility in water and diffuses more rapidly across membranes than DiO, making DiI more useful for labelling the LBL tract.
2) DiI is in a crystal form while DiO is in a smaller flaked form, making DiO more specific and selective for dye filling individual cells and therefore limit dye spread to other areas.
3) DiI fluoresces a Red-Orange when viewed, while DiO fluoresces Green.

The only other known report of the use of the carbocyanine dyes DiI and DiO in a cephalopod was that used to label motor nucleus in the octopus brain (Robertson et al, 1993).

The main advantage and purpose for using these dyes are that;

1) Unlike Lucifer Yellow they can be used in fixed tissue.
2) Because of this, long term dye-filling can be achieved, which is useful for tracing long tracts such as the LBL tract.
3) Because the dye is applied in a crystal form, surface laying tract such as the LBL tract can be dyed with ease.
4) They have a high MW (934 & 882) so dye is resistant to crossing gap junctions.
5) They are not toxic and can stay in the cell without harm over several years (Kuffler, 1990).

DiI was applied to fixed brain slice in the following way:

After the slice had been fixed overnight in 2.5% paraformaldehyde in phosphate buffer, the slice was pinned out in a Sylgard petri dish, excess fixative was poured off and the preparation blotted dry with tissue. The blotting improved the adherence of the crystal to the tract as it became sticky with drying. A small crystal of DiI was applied with fine forceps or a syringe needle to the surface of the LBL tract. Placement of the crystal was the most critical part and required precise handling as to avoid dye spreading to other unwanted brain regions making interpretation difficult. After dye placement, the slice was then re-immersed in fixative, covered and left in the dark at room temperature for as long as required. DiI travelled along the lateral basal lobe tract towards the PCL neuropil within 2 days and into the LBL itself after 7 days of application. The preparation was checked daily for progress.

DiO was applied to individual LBL cells in small flakes. The dye then dispersed over the slice. Cells were stained randomly and quickly in live slices and were seen as a fluorescent bright brilliant red colour when viewed. This technique was easier and more effective for dye filling LBL cells than Lucifer Yellow because of the small cell size and hence difficulty to dye fill using iontophoresis.

Slices dyed with DiI and DiO were cleared using glycerol, viewed and photographed immediately as clearing with glycerol dissolves the dye very quickly and spreads the dye throughout the slice. Clearing the slice with glycerol revealed more detail of the fibres of the tract and cells. DiI preparations were observed with an epifluorescence microscope fitted with a Rhodamine filter set (excitation 530-560nm; emission 580nm), while for DiO an excitation filter of 450-490nm and emission filter of 520nm was used. DiI fluoresces bright red-orange and DiO fluoresces green. DiO fluorescence cannot be seen with rhodamine filters, but the excitation spectrum of DiI is very broad and it produces a goldish fluorescence when viewed with the DiO filters, offering the advantage that they can both be viewed through the same filter set.

Material was examined using a Nikon Optiphot microscope. Photographs were taken on 35mm 100 and 400 ASA colour film, 400 ASA transparency film, and 400 ASA black and white film. The colour film was processed commercially using C41 processing.

2.7 Topical application of neurotransmitters

A variety of drugs were bath applied to the brain slice: acetylcholine (ACh), carbachol, 5-hydroxytryptamine (5-HT), DL-2-amino-4-phosphonobutyric acid (AP-4), hexamethonium dichloride (HEX), tubocurarine (TUB), phentolamine (PHEN) (all from Sigma), gamma-D-glutamylaminomethylsulphonic acid (GAMS), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (both from Cambridge Research Biochemicals), methylsergide maleate, and dihdyro-B-erythroidine (DBE) (both from Research Biochemical Incorporated). The effect of these drugs on the spontaneous tonic activity, synaptic input, and field potential evoked by stimulating the lateral basal lobe tract was tested. In all drug applications, the standard ASW was replaced with an ASW solution containing the drug being tested. Concentrations of the drug were made from a stock solution of 10^{-1}M and then serial dilutions were made down to concentrations as low as 10^{-7}M. The dilution factor of the bath (5ml) was taken into consideration during drug application. The highest concentration of drug

used was 10^{-3}M and the lowest was 10^{-7}M. The lowest concentration was always applied first, then, if no response was obtained, increasing concentrations were successively applied until an effect was observed or 10^{-3}M was reached. Each concentration of the drug was washed out thoroughly through the slice before the next concentration of drug was used. When an effect was observed, the drug was then replaced with the normal ASW and a recovery awaited. It was found that direct application of the drug to the bath by using a Gilson pipette produced good results without interfering with the extracellular and intracellular recordings.

2.8 Analysis of results

2.8.1. Extracellular field potentials

Individual field potentials (FPs) were displayed on an oscilloscope and amplified with conventional preamplifiers. The signal was then sent to a Cambridge Electronic Design (CED) 1401 controlled by the CED 16 channel signal averager programme and PC 486 computer. Multiple traces of one field potential were collected on the computer so that an average of the traces could be made.

The average reading of the field potential was then loaded onto a Sigma Plot programme on a PC 486 and the potentials were displayed as seen in the thesis. To establish the location of the FP recordings within the slice, the slice was drawn so that the location of the FP could be marked. After sufficient FPs had been obtained the pallial nerve (PN) or lateral basal tract (LBL) was dye filled by retrograde transport (section 2.5.3.) to relate the morphology of the pathway of the fibers to the location of the FP recorded. The data was then represented as a photograph of the slice showing the dyed PN or LBL fibers together with actual FP recordings obtained from the adjacent dyed fibers.

By plotting the amplitude of the various field potentials recorded throughout the slice against the distance of the FP from the PN, a contour surface map was made, showing the extent of the field potentials in the slice, in a three

dimensional pictorial representation. The contour map was constructed by placing a grid over a photograph of a PCL slice, from which the field potentials were recorded and their location on the slice marked, as mentioned above. The amplitude of each response was measured from its baseline to its peak, which was then plotted against the distance that the FP was recorded from the site of stimulus, in this case, the PN. These values were then processed using the program Surfer.Version 5.02, from Golden Software Colorado on a PC 486, which plotted the response as a contour map.

2.8.2. Intracellular recordings

The location of a cell within the PCL was either identified by a mark placed on a drawing of the slice or the cell was dyed filled by iontophoresis as discussed earlier.

To facilitate the analysis of the electrical properties and the morphology of cells, a data recording sheet was devised on which the cell location, size, resting potential, electrical activity, spike amplitude, antidromic response, and response from lateral basal lobe stimulation were routinely recorded. The pallial nerve and lateral basal lobe (LBL) tract were stimulated by extracellular bipolar electrodes to evoke extracellular field potentials. A fine pair of Teflon coated, silver wire, electrodes were used to stimulate the LBL tract. This type of electrode provided good focal stimulation and limited the current spread.

The electrical recordings of individual cells were displayed very much the same as for extracellular recordings except that an average of the signal was not taken. The signal was sent to a Cambridge Electronic Design (CED) 1401 controlled by the CED 16 channel signal averager programme and PC 486 computer. Intracellular recordings were loaded form the CED to a PC where the Sigma Plot programme was used to draw graphs and pictures of the traces. The electrical traces seen thoughout the thesis are actual recordings and are not tracings or drawings of the activity. The data was taken directly from the CED computer and transported to the Sigma Plot programme for editing, and text etc.

2.8 3 Morphology

Cell soma diameter, of dyed filled cells, was measured using a graticule insert on the epifluorescence microscope. The number of dendrites on the cell were counted directly from the image seen using the microscope and recorded on the data sheet. Cell morphology was recorded by 1) photography 2) drawings from the microscope image using a camera lucida.

Two silver stained sections of the PCL showing cell size difference (Fig. 4.4 & 4.5) were kindly supplied by Dr. John Messenger of Sheffield University.

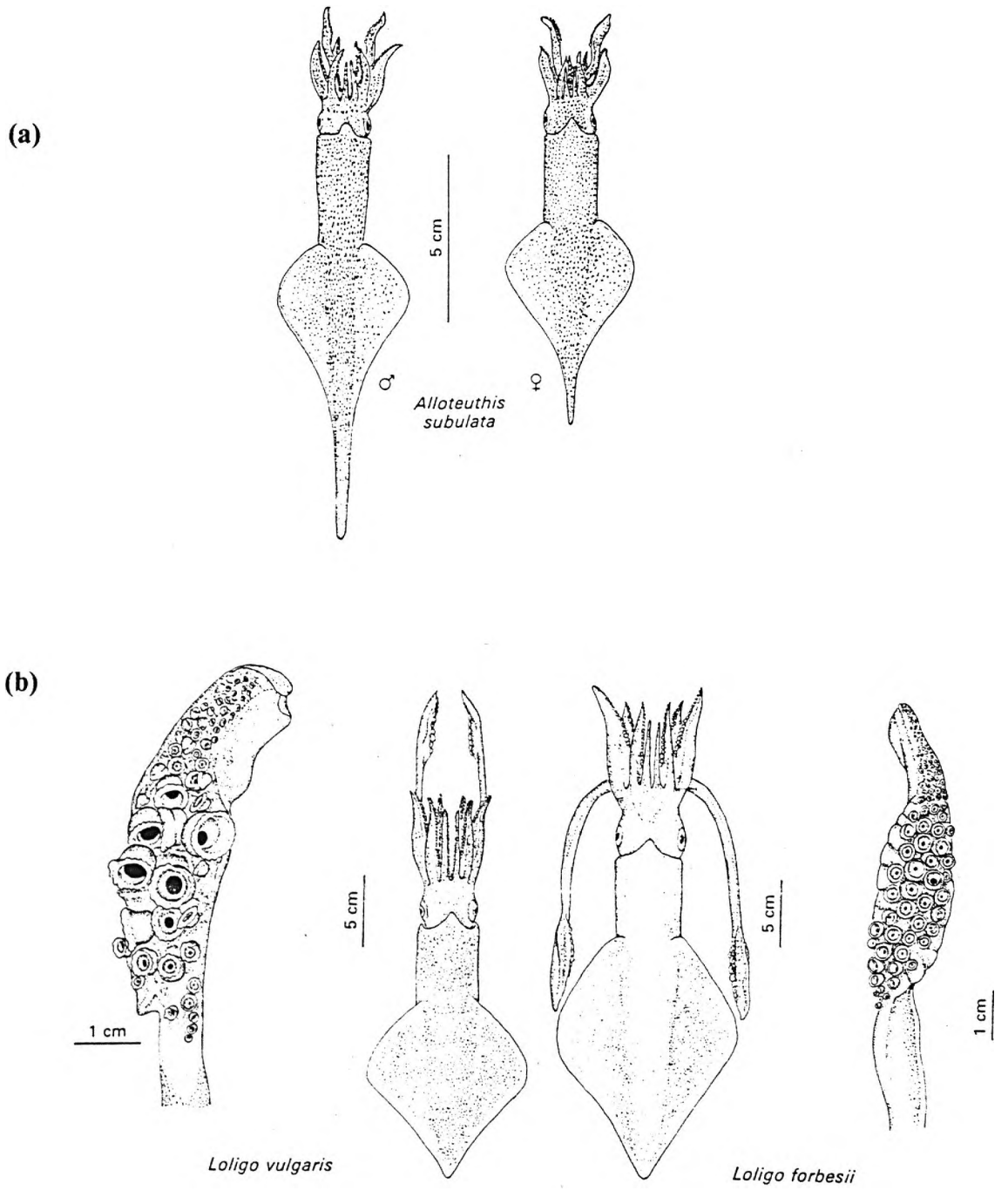


Fig 2.1 Drawings of the three squid species that were identified during the investigation. (a) A dorsal view of a male and female *Alloteuthis subulata* showing the differences between the sexes, especially the elongated fins of the male. (b) A dorsal view of *Loligo vulgaris* and *Loligo forbesii*, clearly distinguished by differences in the sucker arrangements of their tentacle club (from Hayward & Robards, 1981)

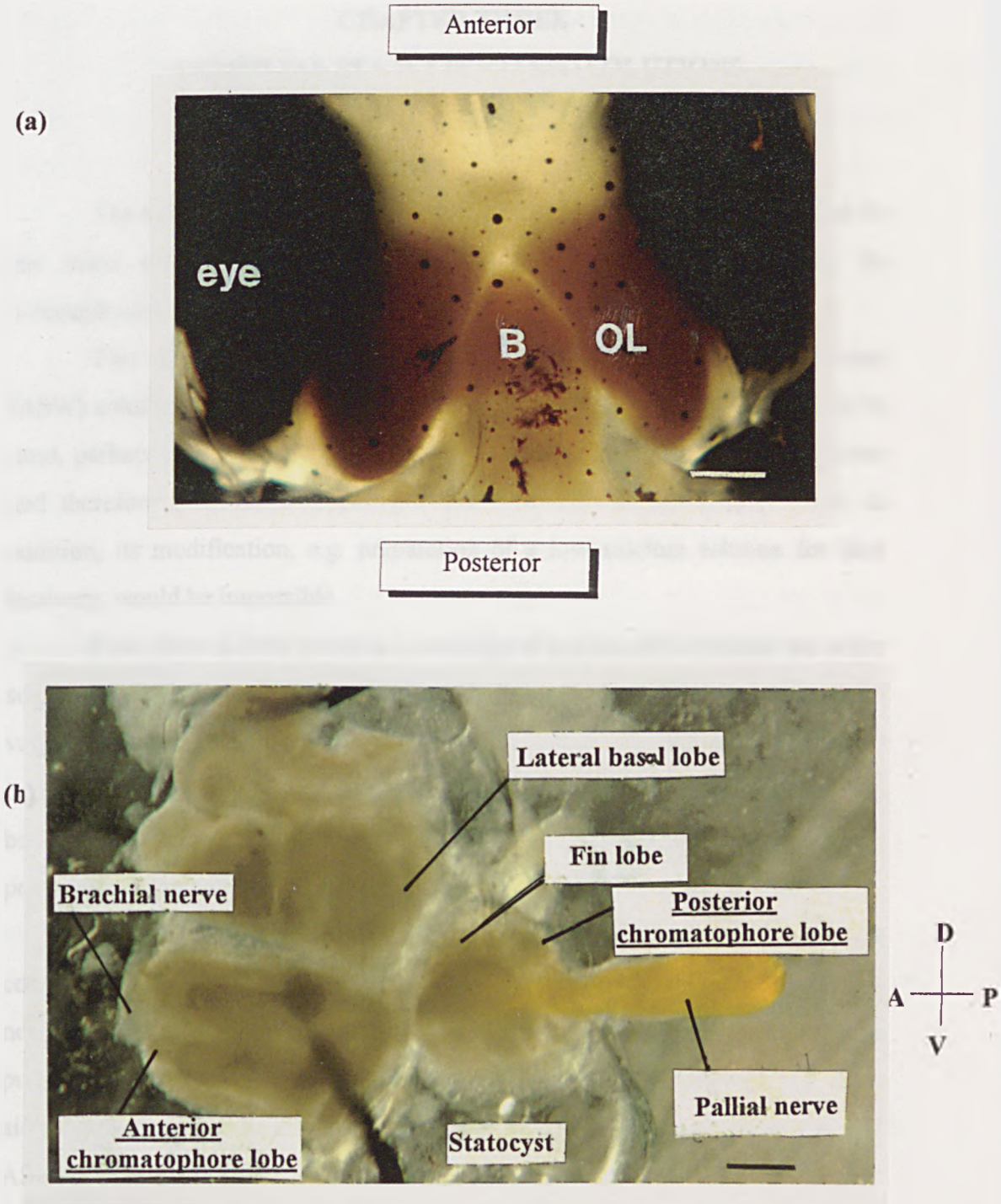


Fig 2.2 (a) A dorsal view of an *Alloteuthis* head before dissection. The eyes, optic lobes (OL), and the brain (B) can be clearly seen thoughout the transparent skin. Retracted chromatophores are visible on the head. Scale bar: 2mm (b) A sagittal brain slice of *Alloteuthis* sliced at 350μm thickness showing the different lobes of the brain and the posterior chromatophore lobes. Scale bar: 500μm.

CHAPTER THREE
ARTIFICIAL SEA WATER (ASW) SOLUTIONS

3.1 Introduction

The aim of this investigation was to provide a suitable bathing solution for the brain slice that would maintain it in a good condition during the electrophysiological experiments.

This is the first report of its kind to test different artificial sea water (ASW) solutions on the cephalopod brain. Although normal sea water could be used, perhaps with the addition of nutrients, its composition would be unknown and therefore difficult to replicate, either over time or by other workers. In addition, its modification, e.g. preparation of a low calcium solution for slice recovery, would be impossible.

Since there is little previous knowledge of a successful artificial sea water solution (ASW) for brain slice preparations, a number of different solutions of varying compositions were tested in order to obtain the optimal solution for experimentation. The criteria used for an optimal solution were; 1) increase in brain survival time 2) improved resting potentials, and 3) improved excitatory postsynaptic potentials (EPSPs).

The solutions have been named 'artificial sea water solutions', for continuity and with keeping with other studies, even though their composition is not the same as that of sea water. When making up a solution, its specific purpose was kept in mind i.e. whether it was to aid recovery after slicing, or to aid long term maintenance of the tissue, as these conditions would need different ASWs. For example the effects of changing $CaCl_2$, NaCl, KCl, and $MgCl_2$ on the slice, were investigated.

A number of ASW solutions were tested during the early stages of the research and their effects on; 1) Field potentials in the optic lobe 2) membrane resting potentials of the posterior chromatophore lobe (PCL) cells and 3) spontaneous and evoked EPSPs of PCL cells in the brain slice were compared.

A previous study, which compared the compositions of the blood and the fluid surrounding the brain in cuttlefish revealed very little difference in their

compositions (Abbott et al, 1985). This finding was borne in mind when we set out to investigate the composition of the fluid surrounding the brain of the squid *Loligo vulgaris*, in an attempt to replicate this fluid for use in the proceeding brain slice experiments.

3.2 Results

3.2.1. Brain jelly solution.

On the assumption that the normal environment of the brain is optimal, the composition of the fluid surrounding the brain within the cranial cartilage, the brain jelly, was chemically analysed (see section 2.3.1.). The brain jelly was found to have a different ionic composition to that of blood plasma sampled from the same species by previous researchers (Robertson, 1953). The major ions, particularly sodium, were at a lower concentration in the brain jelly than in the plasma (Table 3.1). This discrepancy could be due to either contamination of the brain jelly or due to the small sample size analysed, which might not provide a true reflection of the composition of the brain jelly. Collection of the jelly, however, was found to be difficult and very time consuming, limiting the amount of samples analysed, 10 animals in total (for full methods see section 2.3.1). Despite the discrepancy it was decided to try an ASW solution based on the measured ionic brain jelly composition and, this was tested on the optic lobe slice and on the PCL cells of the brain slice preparation.

3.2.2. Effects of brain jelly solution versus Hodgkin ASW solution on field potentials.

The viability of the brain jelly ASW solution was compared with one used by Hodgkin and Katz (1949) for squid axon experiments (for full methods see section 2.3.2). Experiments were performed on the optic lobe slices of *Loligo* since, at the beginning of the study, it was easier to obtain electrical recordings from this type of slice. Extracellular field potentials recorded from the outer plexiform layer were used for monitoring the evoked responses. Field potentials were generated by stimulating an optic nerve bundle.

A field potential in the optic lobe slice was held while the Hodgkin ASW was purfused into the bath, which contained the brain jelly ASW solution. The field potential trace was monitored for changes in its amplitude over a period of 20 minutes. After 10 minutes perfusion of the Hodgkin ASW, the first deflections on the recorded trace, which probably represent field potentials generated by the photoreceptor axons, and a second positive peak, which probably represents synaptic activity between the photoreceptor axon terminals and the second order neurons, both decreased in amplitude (Fig. 3.1b).The field potentials showed signs of recovery after the Hodgkin ASW solution was replaced with the original brain jelly ASW solution (Fig. 3.1c). It is likely that the reduction in the field potentials seen, is indicative of a reduction in the activity of the optic lobe neurons.

A similar experiment was performed but this time the slice was kept in the Hodgkin ASW and then perfused with the brain jelly ASW. Again the effect of these solutions on field potential amplitude was monitored at 5 minute intervals over a period of one hour during which the same field potential was kept (Fig.3.2). As expected from the results obtained above, Hodgkin ASW now generated a gradual increase in the amplitude of the evoked field potential response over time, which is likely to represent an increase in activity of the neurons. However, after about 30 minutes into the perfusion there was a steady decrease in the field potentials (Fig. 3.2). This could be caused by deterioration of the slice by damage to the optic nerves through the continual electrical stimulation, or from movement of the recording or stimulating electrodes during perfusion. In comparing the activity of groups of optic lobe neurons it would appear that the ASW used for squid axons was not as good at preserving the activity of neurons as the brain jelly ASW solution.

With an improvement in the brain slice technique, the brain jelly ASW solution and a tissue culture solution used for squids (Rice et al, 1990) (section 2.3.3) could be tested on the membrane resting potentials (MRPs) of the posterior chromatophore lobe (PCL) cells.

3.2.3. Brain jelly ASW solution and resting potentials in PCL cells.

Given the superior performance of the brain jelly ASW during extracellular recordings in the optic lobe slice, it was tested on the chromatophore lobe brain slice preparation. Intracellularly recorded membrane resting potentials (MRPs) were monitored from PCL cells kept in the brain jelly ASW solution as an indicator of the overall health of the slice and of the individual neurons (for full methods see section 2.3.5.). Under these conditions the average MRP was -22 ± 5.4mV (n=50) for cells in the posterior chromatophore lobe of *Alloteuthis.*

Since these resting potentials are undoubtedly lower than those obtained by other workers, (Williamson & Budelmann (1991) -40 ± 2 mV (n = 42) and Miyan & Messenger (1995): between -20 and -40 mV), a new ASW solution was tested based on a formula used for squid tissue culture which had been reported to improve the membrane resting potentials of cells (Rice et al, 1990,) (for full methods see section 2.3.3).

3.2.4. Tissue culture ASW solution and resting membrane potentials in PCL cells.

This new solution gave an average MRP of -32.2 ± 3.5mV (n = 60) with the slice remaining active for up to 5 hours. This solution therefore, yielded more stable and generally higher resting potentials then the brain jelly ASW (-22 ± 5.4mV, (n=50).

Although the tissue culture ASW gave improved MRPs, these were still lower then those obtained by others, see above.

3.2.5. Effects of $CaCl_2$ on membrane resting potentials in PCL cells.

To improve on the results obtained from the tissue culture solution, $CaCl_2$ was lowered from 10 to 2mmol^{-1}. This solution, comprising of the tissue culture ASW with reduced $CaCl_2$ was used for the slicing and recovery of the brain while the normal tissue culture ASW solution was used for perfusing the recording chamber (section 2.3.3.). The reasoning behind this was that during slicing a certain amount of tissue damage was inevitable. This releases enzymes which can damage intact cells. However, since many enzyme activities require calcium, the

damage can be limited by reducing the available extracellular calcium. Using this modified solution on brain slices proved successful, yielding an average MRP of -37 ± 3.0 mV (n = 45) and with spontaneous activity in the form of EPSPs and action potentials. This slice remained in good condition, (MRP up to -35mV), for up to six hours. This protocol was used during a further 10 animal experiments in order to see if the recordings of membrane resting potential could be improved. But a further improvement in the membrane potential was not observed.

There remained the possibility that the newly found success using reduced $CaCl_2$ in the tissue culture ASW solution for the slicing procedure was due, not to the new solution, but to the improvement in the slicing technique itself. To test this, further experiments were performed using the tissue culture ASW without reduced $CaCl_2$ for slicing and recovery. There appeared to be no difference in the membrane resting potentials obtained after switching back to the tissue culture ASW solution for slicing: -37.2 ± 3.6 mV (n = 55). It could be that the slicing technique had improved rather than the new solutions having an advantageous effect on the slice.

3.2.6. Effects of NaCl on membrane resting potentials in PCL cells.

After 5 months, the tissue culture ASW solution was modified by reducing NaCl by more than half, from 430 to 200 $mmol^{-1}$, and substituting sucrose for NaCl to maintain the osmolarity. This solution was used for dissection and slicing (Table 3.1). The reasoning behind the change was that low NaCl concentrations have been reported to be advantageous for preserving viability of motoneurons in vertebrate brain slices (Aghajanian & Rasmussen, 1989). Neurons are prone to depolarization during anoxia, a condition which is virtually unavoidable during the early phases of slice preparation (e.g., decapitation, blocking, and slicing). This results in acute neurotoxicity whereby a passive influx occurs, followed by cation and water entry. This can lead ultimately to cell swelling and lysis. The replacement of NaCl by sucrose, a relatively impermeable molecule, appears to prevent this type of cellular damage (Aghajanian & Rasmussen, 1989). When first used, this solution appeared to

produce very good results, giving action potentials of 50 mV and MRPs of -40 ± 2.3 mV (n = 46). To test if this success was due to improved techniques or due to the solution itself, the original recipe for the tissue culture ASW solution with normal NaCl (430 $mmol^{-1}$) was used. Again no difference in the MRP was apparent between the two. The original tissue culture ASW solution was therefore used for the slice recovery and perfusion.

3.2.7. The effects of KCl on membrane resting potentials in PCL cells.

To improve on the MRPs of the cells, the tissue culture ASW solution was modified by reducing KCl from 10 $mmol^{-1}$ to 8 $mmol^{-1}$ (Table 3.1). With a decrease in KCl one would expect a rise in the cell MRP but there was no significant change.

3.2.8. The effects of KCl on the excitatory postsynaptic potentials (EPSPs) in PCL cells.

It was important for this investigation to have a solution that promoted EPSP activity in PCL cells. EPSPs being an indication of cell synaptic transport, it was important to work with a solution that preserved this communication.

By increasing KCl concentration the cell can be slightly depolarised according the Nernst equation (see section 2.3.5.) and we wanted to see if this would improve the spontaneous activity of the cell (especially EPSPs). The effects of increasing the KCl concentration of the tissue culture ASW solution were tested during an experiment in which a PCL cell displaying spontaneous EPSP activity, (described in detail in section 2.4.1), was held for 45 minutes. The concentration of KCl was increased from 4 $mmol^{-1}$ to 8 $mmol^{-1}$ and average amplitude and frequency of EPSPs were monitored every 5 minutes (Fig 3.3.).

Increasing KCl concentration caused an increase in the frequency of EPSP events but had no recognisable effect on the amplitude of the EPSPs (Fig. 3.3). This was likely to be caused by a short term shift in the MRP of the cell towards the positive (a depolarization) whereby K^+ ions moved into the cell, causing depolarization of the cell membrane, consequently increasing the spontaneous

activity of the cells. The tissue culture ASW solution was therefore modified to contain 10 $mmol^{-1}$ KCl.

3.2.9. The effects of $MgCl_2$ on EPSP activity in PCL cells.

The idea behind testing $MgCl_2$ was that lowering extracellular magnesium should lower its competitive effect on calcium transport and hence increase synaptic efficiency. The precise mode of action of Mg^{2+} salts on cell membranes in marine animals is poorly understood at present (Messenger et al, 1985), although there is evidence to indicate that high Mg^{2+} levels can effect the post-synaptic membrane at the nerve-muscle junction in crustaceans and vertebrates by competing with calcium receptor sites (Katz, 1966).

The effect of $MgCl_2$ on synaptic activity was tested by lowering its external concentration from 50 $mmol^{-1}$ to 2 $mmol^{-1}$ to see if this would improve the level of EPSP activity. The effect of this solution was tested on field potentials in the optic lobe slice, evoked by stimulating an optic nerve bundle (Fig. 3.4a). A field potential was obtained from a slice kept in the tissue culture ASW solution, and a potential was obtained that had an axon spike and a synaptic component (Fig 3.4). By lowering the $MgCl_2$ concentration, an increase in the amplitude of the axon spike occurred, while the synaptic component was inhibited (Fig. 3.4b). It, thus, appears that lowering the $MgCl_2$ to 2 $mmol^{-1}$ had a depressive effect on the synapse but at the same time potentiated the axon response. The potential started to recover when washed for 10 minutes with 50 $mmol^{-1}$ $MgCl_2$ (Fig. 3.4c).

As already mentioned, $MgCl_2$ competes for, and blocks the voltage-dependent Ca^{2+} channels in the presynaptic nerve terminal. By raising the extracellular $MgCl_2$ from 50 to 120 $mmol^{-1}$ we should be able to block and hence identify a synaptic input. The effects of increased $MgCl_2$ were tested in PCL cells by monitoring the amplitude changes of a field potential over time under perfusion of $MgCl_2$. The axon component of the response remained unchanged, while the synaptic component was depressed, therefore confirming that this

potential was an EPSP (Fig. 3.5b). This blocking effect was partially reversed with washing (Fig 3.5c).

3.2.10. The effects of $MgCl_2$ and $CaCl_2$ on EPSPs in PCL cells.

The combined effects of increasing the $MgCl_2$ and lowering the $CaCl_2$ concentration might give an even more potent inhibitor and hence a better indicator of synaptic activity. By increasing the $MgCl_2$ concentration and lowering the $CaCl_2$ concentration, the frequency of spontaneous EPSPs decreased. This effect was reversed on washing, and there appeared to be no recognisable effect on the amplitude of the EPSPs (Fig. 3.3). Therefore, low $CaCl_2$ and high $MgCl_2$ affects the presynaptic cell by effecting the number of impulses arriving at the postsynaptic cell membrane. $CaCl_2$ is known to be required for the presynaptic release of neurotransmitter and $MgCl_2$ blocks Ca^{2+} receptor binding sites. This result agrees with findings of other researchers (del Castillo & Stark, 1952). Based on these findings, it was decided to leave the $MgCl_2$ concentration in the tissue culture ASW at 50 $mmol^{-1}$ as this did not appear to effect synaptic transmission, nor any other cellular processes.

3.2.11. Williamson & Budelmann ASW.

To improve on the results gained from the tissue culture solution, an ASW solution previously used for electrophysiological work on cephalopod brain slices was used (Williamson & Budelmann, 1991). There was no significant improvement in the MRP or the electrical activity of the PCL cells when using this solution.

3.2.12. The final test for the ASW solutions.

As a final test of slice viability, the slice was incubated in the squid tissue ASW solution containing the fluorescent dye, fluorescein-di-acetate (FDA). If the cells were in good condition they would metabolize the ester form, FDA, and release fluroscein into the cytoplasm. This can be observed as green cells by using a fluorescent microscope (Fig. 3.6). This proved successful, and therefore

the final solution remained unaltered as a modified version of that used for squid tissue culture but with lower KCl. For a summary of the different ASW solutions used see table 3.1.

3.3 Summary

1) The brain jelly from 10 specimens of *Loligo vulgaris* was analysed and its ionic composition was established.

2) An ASW solution based on the ionic composition of the brain jelly measured, was tested on the optic lobe slice and PCL brain slice. A number of other ASW solutions were also tested on the brain slice for comparison.

3) The viability of the different ASW solutions was tested with respect to their effect on field potentials in the optic lobe and brain slice, on membrane resting potentials in the PCL, and their effect on EPSP activity displayed by the PCL cells.

4) In particular, the effects of reducing NaCl and $MgCl_2$ concentration and increasing KCl concentration were tested on brain slice viability.

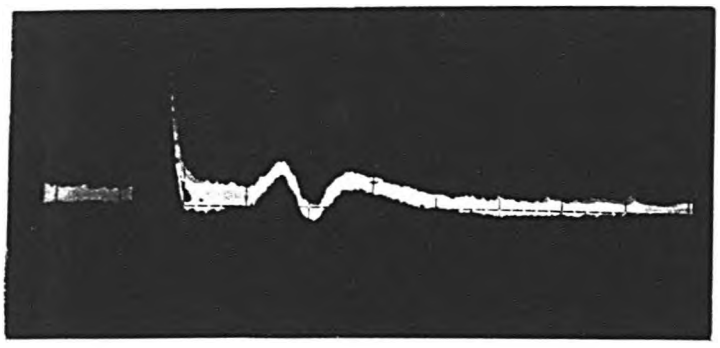
5) A final test for viability of the brain slice under various ASW solutions, was to add FDA, a dye which is metabolised by healthy cells to produce a green fluorescent light.

6) The ASW solution found to maintain the brain slice in the best condition was a modified version of that used for squid tissue culture: in $mmol^{-1}$, NaCl 430, KCl 10, $MgCl_2$ 50, $CaCl_2$ 10, HEPES 20, Glucose 10. This solution was therefore used for all the following experiments because it gave consistently high membrane resting potentials (37.2 ± 3.6 mV, $n = 55$) and the slice survived the longest. Up to eight hours of viable recordings were obtained from PCL cells, under this solution.

7) An important conclusion gained from using the different ASW solutions to maintain slices, was that the preparation of the animal and the improvements in the brain slice technique were as critical for the overall health of the slice and consequently PCL cell recordings, as changes made to the ASW solutions.

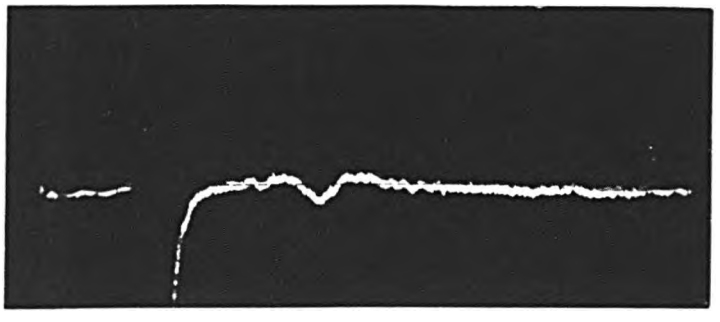
(a) Before

In Brain Jelly ASW



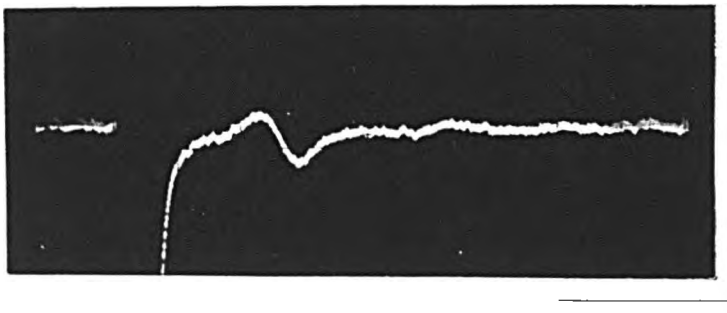
(b) During

In Hodgkin ASW



(c) After

In Brain Jelly ASW



10 msec

5mV

Fig 3.1 Comparison of extracellular field potentials in a range of ASW solutions. (a) An extracellular field potential, evoked by the stimulation of the optic nerve, recorded from the optic lobe slice of *Loligo vulgaris*. This field potential was taken from a slice immersed in a solution of artificial brain jelly, and before the perfusion of the Hodgkin ASW. The initial field potential can be seen in (a). After the perfusion of the Hodgkin ASW the field potential was depressed (b) and after washing the preparation with the brain jelly solution a slight recovery could be seen (c). The polarity of the stimulus artefact has been reversed in (b) & (c) to limit damage to the same optic nerve during continuous stimulation.

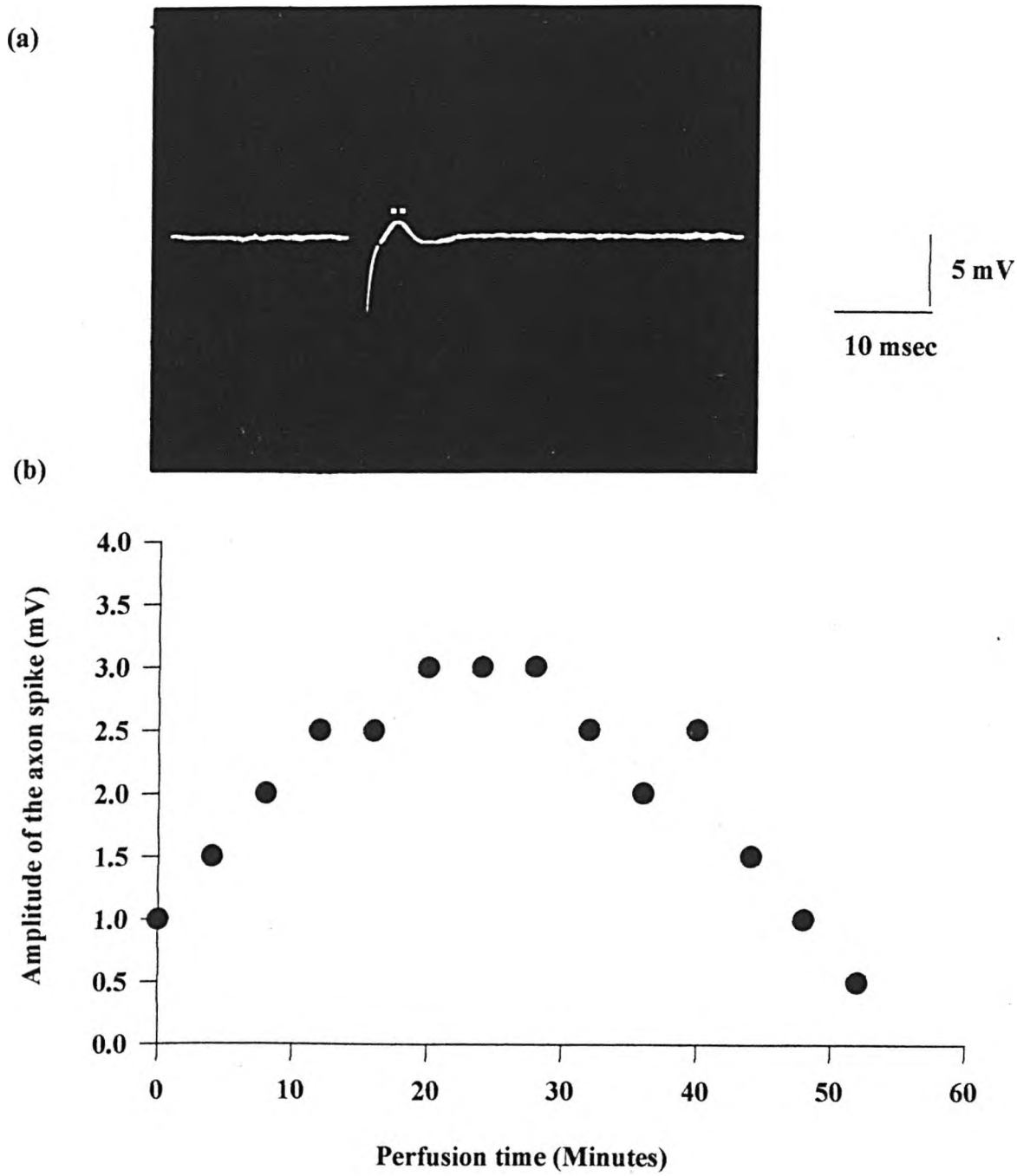


Fig 3.2 The effect of brain jelly ASW on the amplitude of an optic lobe extracellular field potential kept in a starting solution of Hodgkin ASW. (a) A field potential with an axon spike (••) from a *Loligo* optic lobe slice immersed in the Hodgkin ASW. (b) Graph showing the initial increase in axon spike amplitude with perfusion of the brain jelly solution, followed by a gradual decrease of the spike amplitude with time.

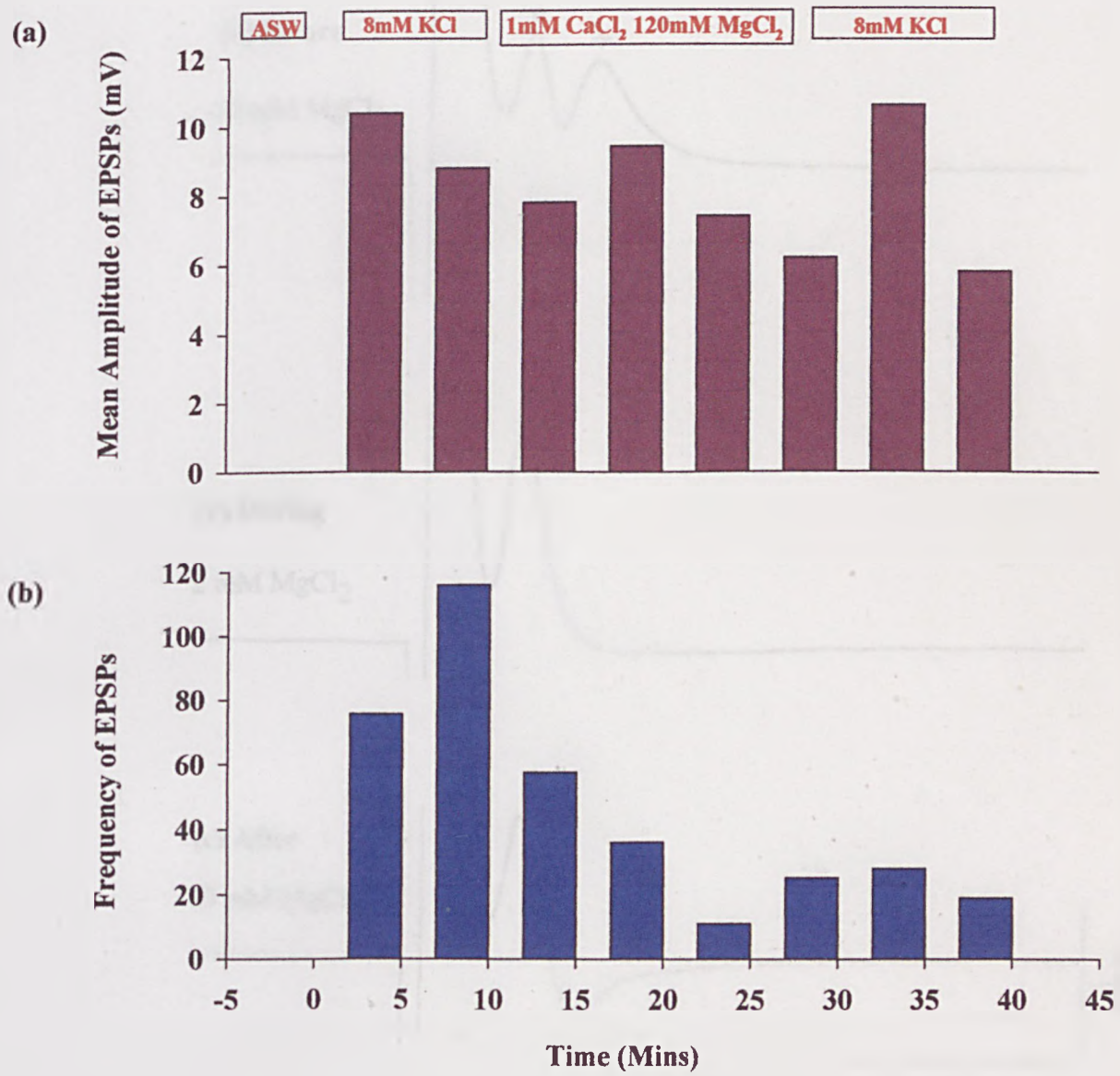


Fig 3.3 Effects of increased bath concentrations of KCl ,and low $CaCl_2$ and high $MgCl_2$ on EPSP amplitude and frequency in a PCL neuron. (a) A graph showing the change in the amplitude of the EPSPs of an *Alloteuthis* PCL neuron with an increase in KCl concentration, and the effects of a low $CaCl_2$ and high $MgCl_2$ solutions. There did not appear to be any significant change in the amplitude of the EPSPs. (b) The EPSP frequency increased with an increase in KCl which was blocked with low $CaCl_2$ and High $MgCl_2$, this effect was recovered with washing.

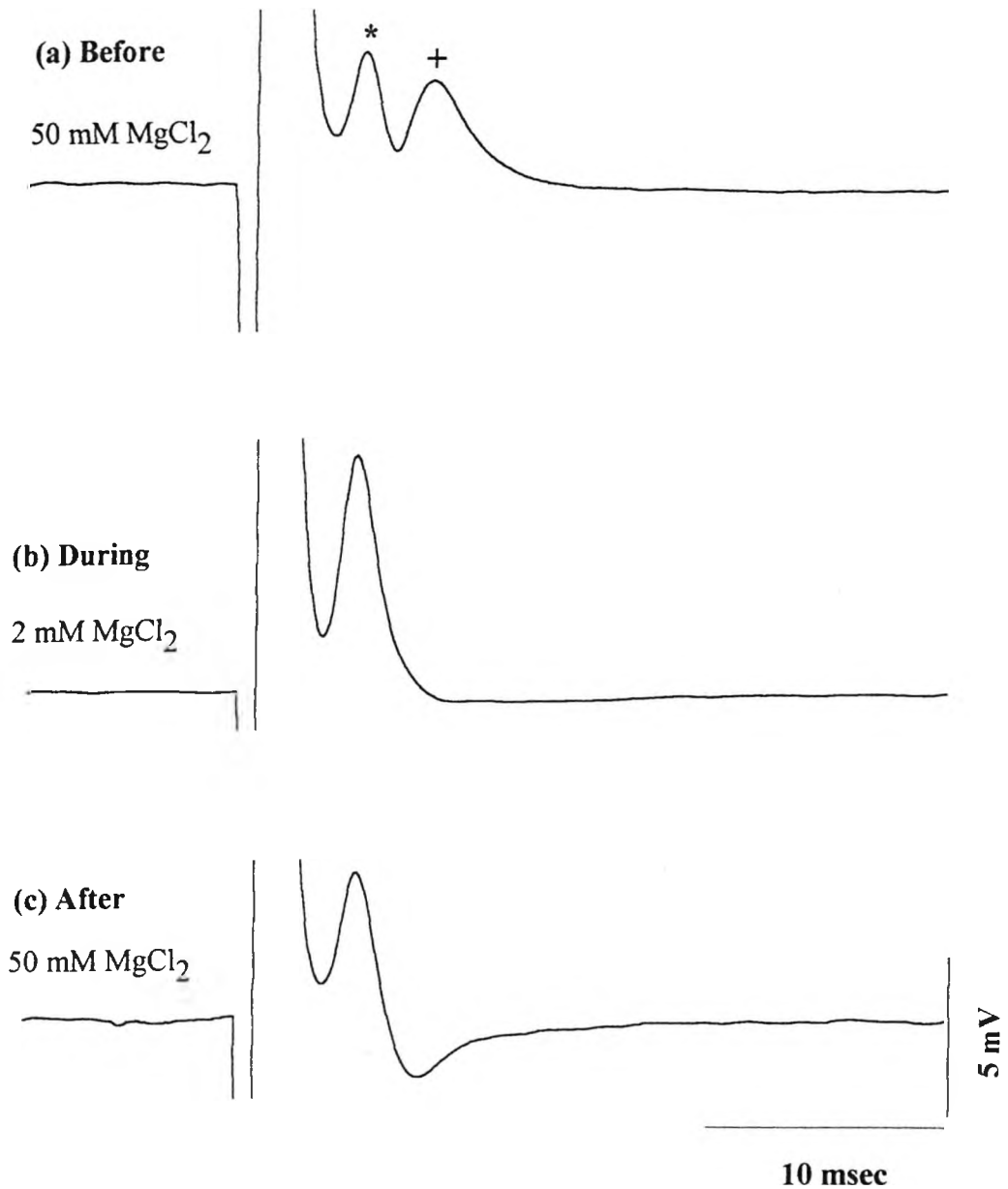


Fig 3.4 Comparison of an optic lobe extracellular field potential in reduced $MgCl_2$. The concentration of $MgCl_2$ was lowered from 50 to 2mM in an attempt to see if this solution would improve the synaptic efficiency of the cell. This was tested on field potentials from the optic lobe slice of *Loligo*. (a) The field potential had a characteristic axon or fibre spike (*) and a possible synaptic component (+). (b) When an ASW solution containing 2mM $MgCl_2$ instead of 50mM $MgCl_2$ was perfused across the optic lobe, the fibre spike amplitude increased, but the synaptic component was depressed. (c) With washing, the fibre spike amplitude decreased but the synaptic component did not recover.

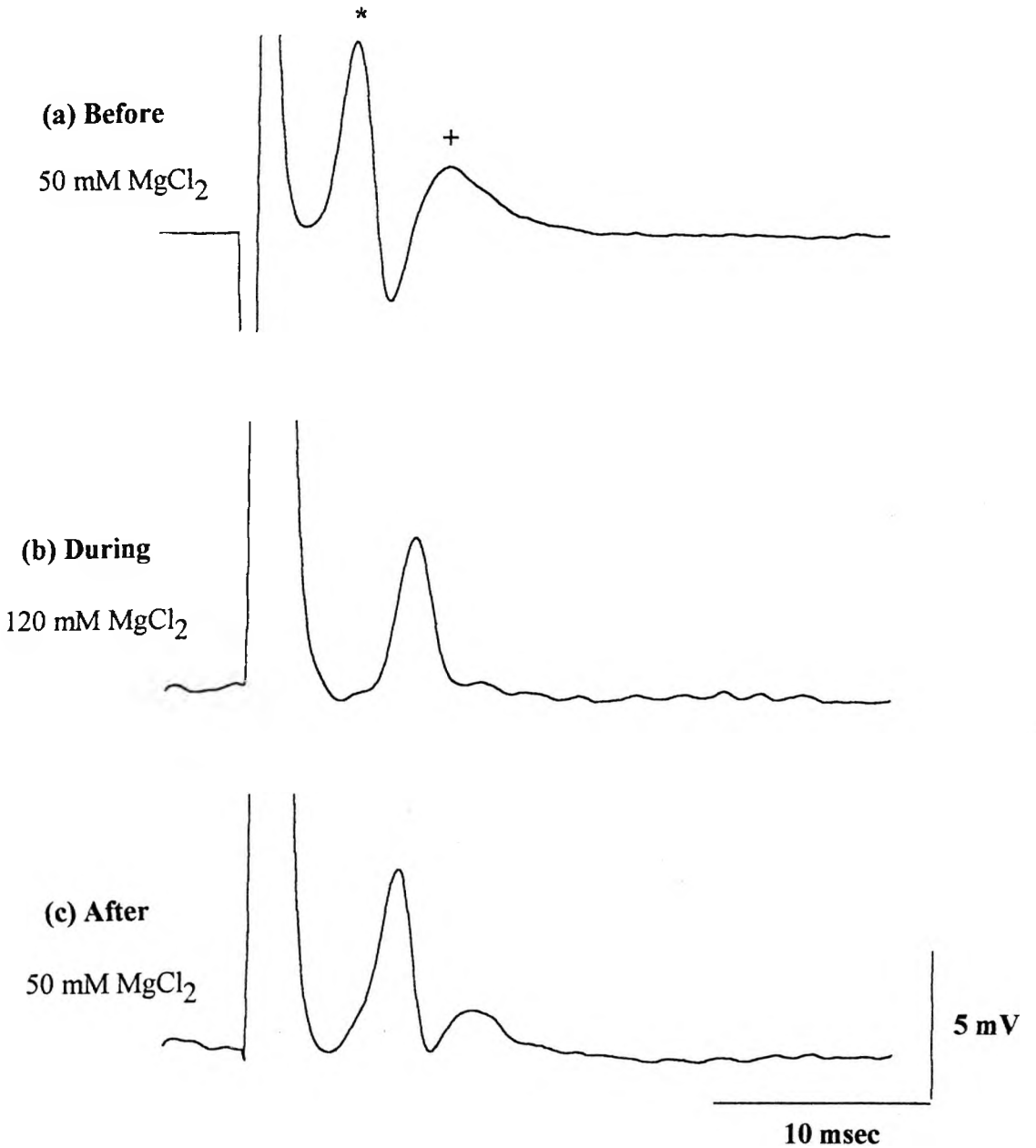
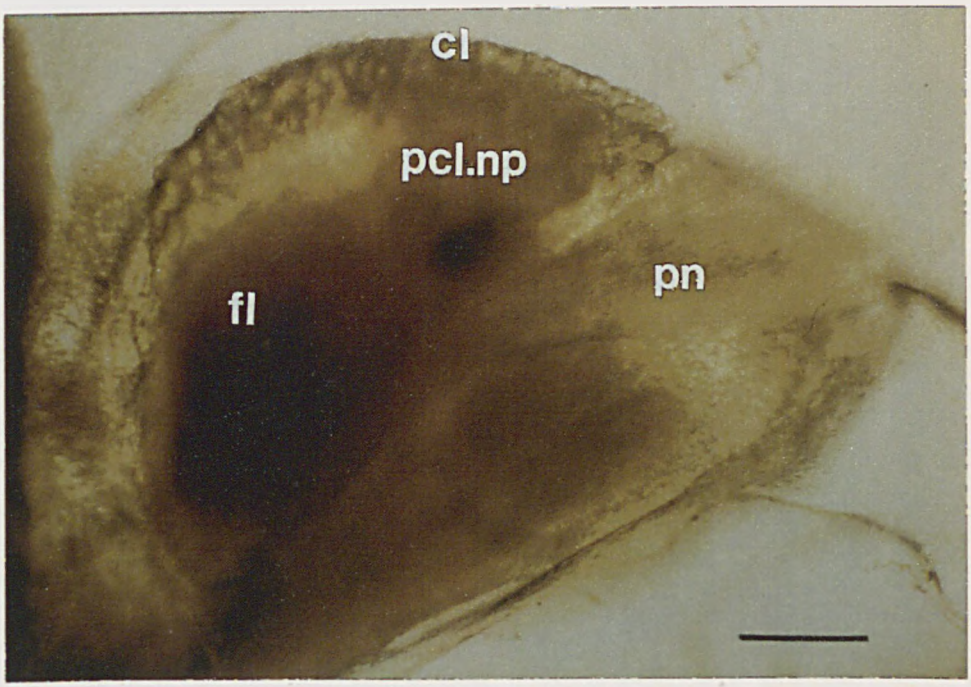


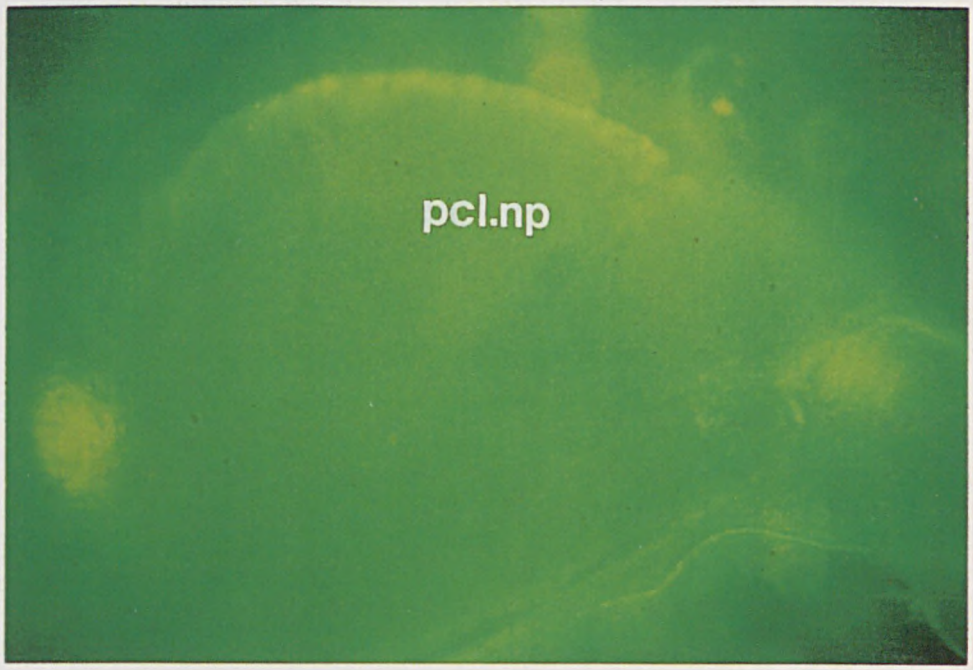
Fig 3.5 Comparison of a *Loligo* optic lobe extracellular field potential in high levels of $MgCl_2$. (a & b) It was found that on application of 120mM $MgCl_2$, compared to an initial concentration of 50mM, the synaptic component of the field potential (+) was depressed while the initial fibre spike (*) remained relatively unchanged. (c) With washing the field potential started to recover with the synaptic component becoming apparent once again.

Fig 3.6 (a) A light microscope picture of a PCL slice from *Alloteuthis*. The PCL neuropil (pcl.np) and cell layer (cl) are visible as is the fin lobe (fl) and the pallial nerve (pn). (b & c) The same PCL slice was incubated in a solution of Fluorescein-di-acetate (FDA) under which cells that were in good condition stained green. When viewed under fluorescent light the cells could be clearly seen to fluoresce green. The slice was cut in a sagittal orientation at a thickness of 400μm. Scale bar: 500 μm (a,b), 60 μm (c).

(a)



(b)



(c)

Table 3.1 Ionic compositions of solutions measured in this and previous studies (italics) and solutions developed and tested in previous studies and in the present study (bold). The final solution used in future experiments is the Artificial Brain Jelly with Low KCl at the bottom of the table.

	NaCl $mmol^{-1}$	KCl $mmol^{-1}$	$MgCl_2$ $mmol^{-1}$	$CaCl_2$ $mmol^{-1}$	HEPES $mmol^{-1}$	Glucose $mmol^{-1}$	pH	Osmolarity ($mOsm\text{-}kg^{-1}$)
Brain jelly Loligo V	296	13.4	38.7	8.4	-	-	-	-
Plasma Loligo V (1)	419	20.6	51.6	11.3	-	-	-	-
Brain jelly Sepia O (2)	464	10.9	50	9.5	-	-	-	-
Plasma Sepia O (2)	464	10.5	51	9.4	-	-	-	-
Artificial Brain Jelly solution	300	14	39	9	10	20	7.5	679
Hodgkin & Katz (3)	470	10	53	11	20	-	-	-
Tissue Culture solution (4)	430	10	50	10	20	10	7.7	800
Low $CaCl_2$	430	10	50	2	20	10	7.7	1000
Low $NaCl_2$	200	10	50	10	20	200	7.8	980
Artificial Brain Jelly with Low KCl	**430**	**8.0**	**50**	**10**	**20**	**10**	**7.6**	**969**

References : (1) Robertson (1953), (2) Abbott et al (1985), (3) Hodgkin & Katz (1949), (4) Rice et al (1990).

CHAPTER FOUR
MORPHOLOGY

4.1 Introduction

The basic anatomy of the posterior chromatophore lobe (PCL) has been investigated in a number of cephalopods (e.g. Young, 1971; Young, 1976). These studies have shown that the PCL consist of a fairly discrete group of cells, and associated neuropil, in the posterior part of the suboesophageal mass. Such work has mainly used standard histological techniques such as tissue sectioning and silver staining, that give gross anatomical information such as the size and position of the cell somata, sometimes show some of the cells dendrites, and possibly part of the axonal process. However, these methods do not reveal the full morphology of individual cells and hence give little information on the shape or size of the cells dendritic tree, or the areas of overlap with other cells that could lead to cell interactions. Only Miyan & Messenger (1995), looking at the octopus PCL, have used modern microelelctrode techniques that permit the injection of dye into a single cell that then diffuses throughout the cell to reveal the cells morphology; however, this work did not set out to identify the cells recorded from as chromatophore motoneurons.

In this section, the squid PCL has been investigated using a combination of standard histology, dye backfills of the pallial nerve, and intracellular injection of fluorescent dye markers into individual PCL cells. This combined approach has revealed the gross anatomy of the PCL, as well as the position, size, shape, and dendritic patterns of single neurons. This information has been analysed to build up a picture of types of cell present in the PCL and, based on the morphology patterns, their likely connections and possible function. In addition, because this work has been combined with electrical stimulation and recording technique (see later) many of the cells filled could be identified as chromatophore motoneurons.

4.2 Results

4.2.1. The posterior chromatophore lobe (PCL).

In general, the basic morphologies of the brains of the squids, *Alloteuthis subulata* and *Loligo vulgaris* are similar (Young, 1976). In both, the PCL forms a clear cell grouping at the back of the posterior suboesophageal mass (Fig.4.1). The PCL is related to the fin lobe, which lies immediately anterior to it, but has no interchange of fibres with it (Young, 1976). The PCL sends most of its output fibres to the ipsilateral pallial nerve, which exists in the brain ventrally to the PCL (Fig.4.1). The pallial nerve is a complex nerve comprising of three major nerves, including the fin nerve (Young,1976). Dye backfills of the pallial nerve into the brain show stained cell bodies within the PCL and fin lobe, together with fibres within the neuropil areas of these lobes (Fig 4.2a). The cells of the fin lobe are generally larger than those of the PCL, being up to 140μm in somata diameter, and all are presumed to be motoneurons (Young, 1976) (Fig 4.2b). The stained cells shown in Figure 4.2 are very closely grouped and vary in their degree of staining, with those most strongly stained being nearer the pallial nerve. Although this could just be due to the sporadic nature of the dye filling, it could also be an indication that there is some degree of dye coupling between these cells (Fig 4.2b). The nerve back-filling method is a convenient way of identifying the chromatophore motoneurons and their output pathway.

In many of the backfills of the pallial nerve, a fibre tract was observed to run ventrally, in association with the pallial nerve; this is probably the posterior head retractor nerve (Fig. 4.3).

The PCL receives its main central input from the suboesophageal lateral basal lobe (LBL), via the lateral basal tract (LBT). This tract could be clearly seen in sagittal brain slice sections (Fig.4.1). The LBL, and its influence on the PCL neurons, is investigated fully in Chapter 7.

The ipsi- and contralateral PCLs are connected to each other by the posterior chromatophore commissure, as shown in Fig 4.4. This commissure is not thought to carry axons from the cells of PCL neurons, but from the LBL to

PCL tract (Young, 1976). The PCL is also connected to the ACL by the dorsal and ventral inter-chromatophore tracts.

4.2.2. Neuron size.

Previous work on *Octopus* and *Sepia* has shown that the PCLs in these animals have larger cells towards their periphery and smaller cells in the central area (Young, 1976). *Loligo* has been reported to have a simpler arrangement with similar sized cells arranged in rows, and no apparent inner layer of small cells (Young, 1976). However, in this present investigation of over 400 cells in the PCL of *Alloteuthis* and *Loligo*, it became clear that the PCL neurons are indeed regularly arranged with the largest cells being at the periphery and the smallest cells being at an inner layer. (Fig 4.5). The cell somata at the periphery are very large, some being up to 25μm diameter, gradually decreasing to smaller cells of 15μm diameter at the inner cell layer. In *Octopus*, the cell somata range in diameter from 20μm to less than 5μm from the periphery to the inner cell layer (Young, 1971), so there is a smaller range of cell size in *Alloteuthis*.

4.2.3. Neuron morphology.

A total of 115 cells in the PCL were stained by intracellular dye injection; these included 96 from *Alloteuthis*, 14 from *Loligo vulgaris*, 5 from *Eledone* and 1 from *Octopus*. The majority of these cells had complete axons which could be traced, on reconstruction, running towards the pallial nerve. The morphologies of the cells stained in *Alloteuthis* and *Loligo*, revealed some general characteristics. These included regular membrane swellings of various sizes and shapes, observed on the dendrites of many cells (Fig.4.6). These swellings could represent boutons or sites of synaptic transmitter release. This type of swelling has also been documented for *Loligo* by Young (1976). Similar swellings have also been documented in *Octopus* PCL neurons, where they have been thought to represent coupling between glial cells and/or between axons of the impaled neuron (Miyan & Messenger, 1995).

In the majority of dyed-filled cells, dendritic branching was seen at the level of the neuropil area. Individual axons gave off branches until they left the lobe, but the most distal branches were usually small. This arrangement undoubtedly exposes the cell to a wide source of inputs, or provides outputs, within a large part of the PCL neuropil. The axons of the cells, which were dye filled in close proximity to each other, took very similar pathways through the neuropil, offering many opportunities for synaptic contact (Fig 4.7). Many cells in the PCL had cell body dendrites as well as axonal processes, which is unusual for invertebrates (Fig 4.7 & 4.15). Soma dendrites have been documented in an *Octopus* whole brain preparation (Miyan & Messenger, 1995) but not until now in the squid brain.

Not all the stained cells had long axons extending towards the pallial nerve and these could not, therefore, be categorised as motoneurons. Some of these cells had short axons, resembling the vertebrate retinal amacrine cells (Fig 4.8). This type of cell could be involved in local signalling within the PCL. An example of this was demonstrated by a star shaped interneuron with short dendrites extending radially outwards (Fig 4.9). A similar type of cell has been found in the octopus PCL (Young, 1971).

Apart from these general observations, there were some striking morphological differences between cells in the PCL of *Alloteuthis*. It was found that cells could be categorised according to their morphology. This observation was made only after the photographs of all the dye-filled PCL neurons were observed and certain morphological similarities among cells became apparent. The word "category" or cell type used in this thesis is based not only on observation but also on quantifiable data which includes the distance of the first axon dendrite or arborizations from the cell soma, the number of dendrites per cell and the location of the cell in the PCL. The most striking difference between the cells was the distance of the dendrites from the cell soma.

Not only did these cells differ in their morphology but they were also located predominantly in certain areas of the PCL (Table 4.1). The location of the dyed filled cells was marked on a diagram of the PCL slice at the end of each experiment. Unfortunately no measurements were taken of the distance of the cell

from the periphery, so the location of the cell could not be quantified, this was because we did not expect to find this topographical relationship. The PCL was however divided into areas spreading from the periphery of the lobe to the neuropil. These areas were called outer, middle, and inner zones, junction with the fin lobe neuropil, and neuropil. (Fig. 4.11). This zonation has not however been substantiated morphologically.

Not every dye-filled cell in the PCL fitted into one of the categories therefore there is some discontinuity between cell types, and other cells are present in the PCL as mentioned earlier. However 83% of the cells dyed filled in the PCL can be divided into the categories mentioned below. The examples of cells distinguished below are representative of each cell category.

The categories of PCL cell types can be divided into representative types:

I. cells with no arborizations (Fig 4.10a).

II. cells with extensive arborization in the cell layers and neuropil (Fig 4.10b).

III. cells with arborizations restricted to the neuropil (Fig 4.10c).

IV. cells with two long processes, possibly axons, emanating from the cell body (Fig 4.10d).

4.2.4. Characteristics of the cell types.

Cell Type I had a relatively simple morphology with few or no visible arborizations; the axon was fine and showed no sudden narrowing or axon hillock (Fig. 4.10a & 4.12). Out of a sample size of 96 dyed filled PCL cells, 10 were of this type and of these 50% were located predominately in the inner cell layer (Table.4.1).

In contrast, cell Type II had a complicated morphology with extensive arborizations that started 10 ± 5 μm (mean ± SD, n = 50) from the cell soma and continued into the PCL and neuropil area. The axon of this cell type was thick, especially at the cell soma 8 ± 2 μm (mean ± SD, n = 50), which then narrowed (Fig 4.10b & 4.13). These cells were the most common of all the cell types,

occurring in 50 of the total cells sampled (n = 96), and 44% of these cells were distributed in the outer cell layer (Table.4.1).

In cell Type III, arborizations appeared some distance 68 ± 8 μm (mean ± SD, n = 33) from the cell soma, generally at the point where its axon entered the neuropil region (Fig. 4.10c & 4.14). This arrangement would maximise the amount of synaptic contact of the dendrites with other cells in the neuropil. Type III cells could receive and transmit impulses not only from the neuropil region but also within the cell body layers of the PCL. The axon of this cell type was thin 2 ± 1 μm (mean ± SD n = 33), and showed no signs of being thicker at the cell soma. This cell type occurred in 33 of the total cells sampled (n = 96), and 64% of these cells were distributed in the outer cell layer (Table.4.1).

Cell Type IV was quite different to any of the other cell types. As well as the axon coming from the cell soma with a thickness of 4 ± 1 μm (mean ± SD, n = 3), a dendrite almost as thick 2 ± 1 μm (mean ± SD, n = 3) also branched directly from the soma together with smaller soma dendrites (Fig. 4.10d, 4.15 & 4.16). This cell was closely associated with the short axoned cell shown in Fig. 4.8 (cf. Fig. 4.16). This type of cell occurred in only 3 of the total cells sampled (n = 96), and each of these three cells was distributed in the inner cell layer, the neuropil, and the at the junction of the neuropil of the PCL with the fin lobe (Table 4.1).

Further investigation of the different cell sub-types showed that the average number of dendrites per cell was;

Cell type I	4 ± 2 (mean ± SD n = 10)
Cell type II	15 ± 4 (mean ± SD n = 50)
Cell type III	11 ± 3 (mean ± SD n = 33)
Cell type IV	10 ± 5 (mean ± SD n = 3)

and this was correlated with the position of the cell soma; i.e. the mean number of dendrites per cell was highest in the outer cell layer 15 ± 5 (mean ± SD n = 44) and lowest in the inner cell layer 5 ± 2 (mean ± SD n = 19) (Table 4.2). This relationship was mainly due to the frequent occurrence of cell Type II (22 n = 44) and III (21 n = 44) in the outer cell layers.

4.2.5. Dye coupling among PCL neurons.

Dye coupling was observed in four out of a total of 95 cells sampled. The cells that were dyed by intracellular injection were brightly stained while the dye-coupled cells did not stain so brightly. The dye coupled cells were always closely associated with each other (Fig. 4.17). Dye coupling could indicate the presence of electrical coupling between some PCL neurons and may be one mechanism by which co-ordination of electrophysiological activity in a sub-population of neurons occurs, i.e. co-ordinating patterns in the skin.

4.2.6 Implications for future work.

Given that there are four distinct morphological cell sub-types within the PCL, based on the distance of the dendrites from the cell soma, the position of the cell, and the number of dendrites per cell, we can then ask if these morphological differences are related to different cell functions or physiology. In an attempt to answer this question, routine intracellular recordings were made from the different cell types as well as from those cells in the PCL which do not conform to the cell categories described. The electrophysiological results are described in detail in Chapter 6.

4.3 Summary

1) The brains of *Alloteuthis* and *Loligo* are similar in morphology: the PCL has its main input from the lateral basal lobe and its main output goes to the ipsilateral pallial nerve.

2) The PCL neurons of *Alloteuthis*, *Loligo* and *Octopus*, are regularly arranged with the largest cells at the periphery and the smallest cells near the neuropil.

3) Some PCL neurons have dendritic branches on the cell body, and not just in the main bulk of the neuropil, perhaps indicating the presence of a special system of excitation.

4) Neurons of the PCL can be divided into four representative categories: Type (I) Cells, with no aborizations. Type (II) Cells, with extensive aborizations in the cell layers and neuropil. Type (III) Cells, with aborizations restricted to the neuropil. Type (IV) Cells, with two long processes, possibly axons, coming from the cell body. These categories of cells were quantified with respect to the distance of the dendrites from the cell soma, number of dendrites and position of the cells in the PCL. There are a few cells which do not conform to this categorisation.

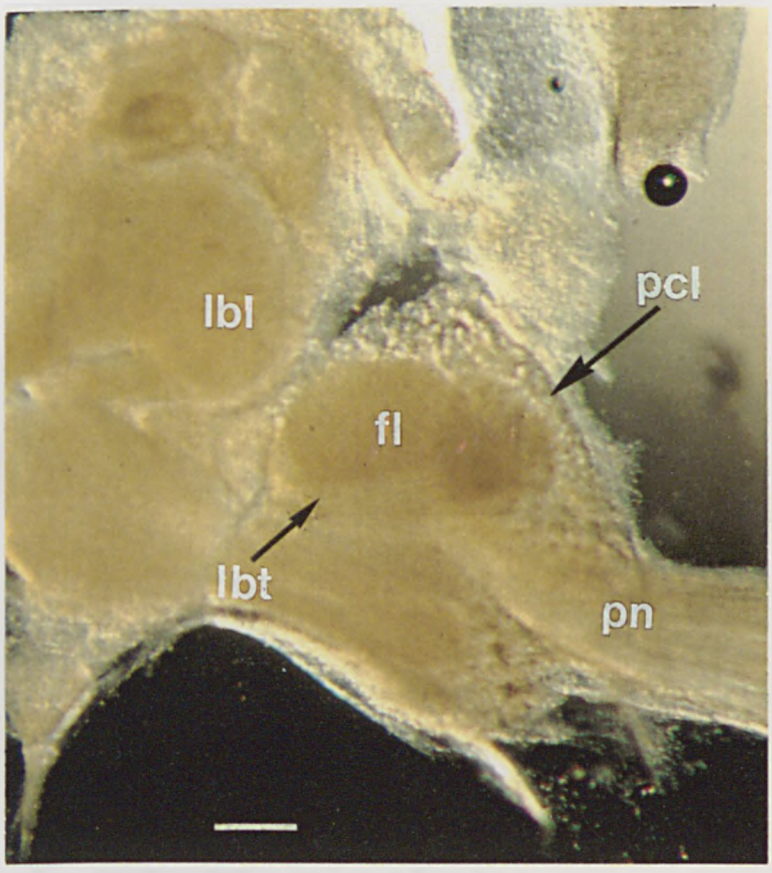
5) These representative cell types are located in separate areas of the PCL: Type (I) cells are located near the neuropil, type (II) cells in the outer and middle cell layers type (III) cells in the outer lobe, and type (IV) cells in the neuropil area.

6) Four out of 95 cells were found to be dye-coupled which could indicate the presence of electrical coupling and may be one mechanism by which adjacent PCL neurons co-ordinate their activity to influence patterns on the skin.

7) These results lead to a number of questions and implications for further research. In particular, whether different cell functions are related to the cell types found and whether or not electrical coupling is present between PCL cells. These questions are addressed in chapter 6.

Dorsal

Anterior

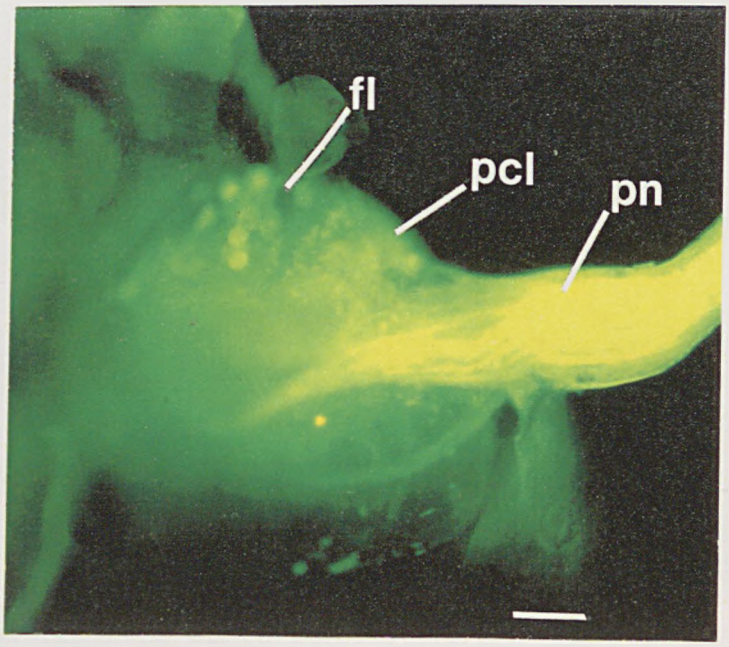


Posterior

Ventral

Fig 4.1 *Alloteuthis* sagittal brain slice showing the posterior chromatophore lobe (pcl), pallial nerve (pn), fin lobe (fl), the lateral basal lobe (lbl), and the LBL tract (lbt). Scale bar: 500 μm.

(a)



(b)

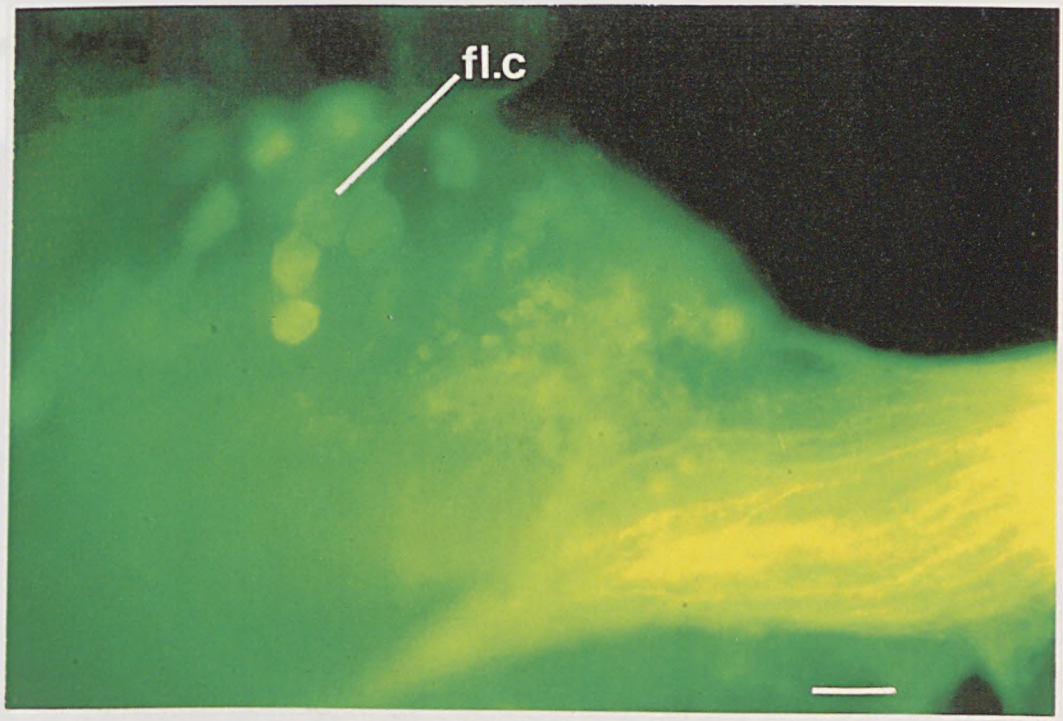


Fig 4.2 (a) A fluorescence photograph of an *Alloteuthis* PCL slice which has had the pallial nerve (pn) stained with LY. The dye has travelled to the PCL and fin lobe (fl) of the slice. Scale bar: 500 μm. (b) Higher power view of (a) showing the large cells stained in the fin lobe, compared to those in the PCL. Note the close association of these cells (fl.c). Scale bar: 250 μm.

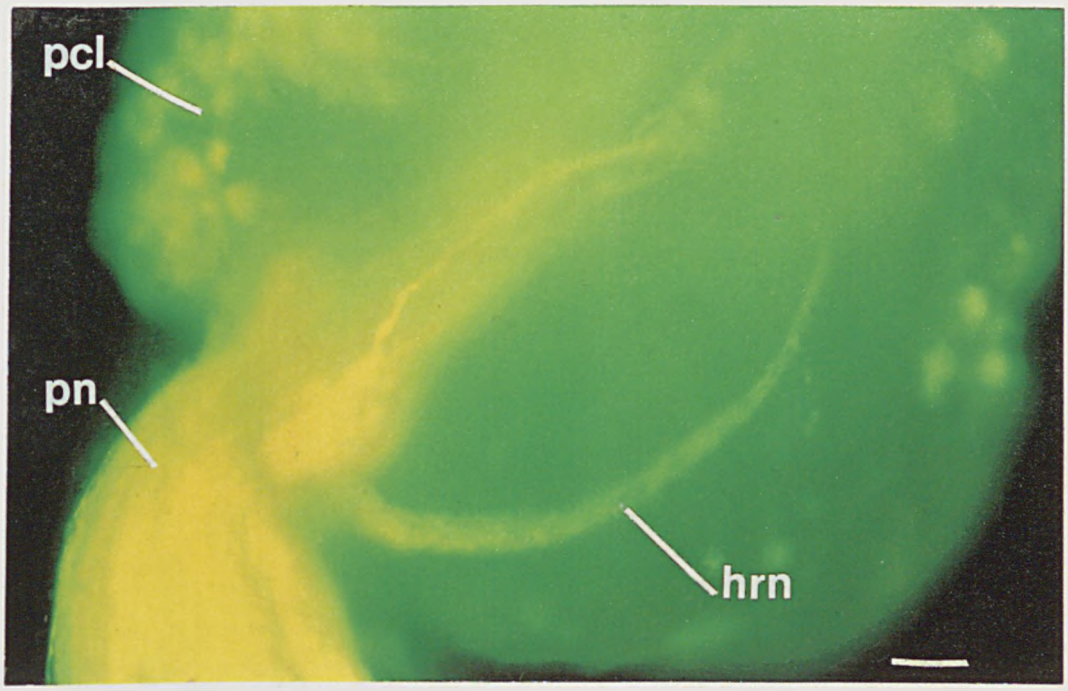


Fig 4.3 A fluorescence photograph of an *Alloteuthis* PCL slice in which the pallial nerve (pn) was filled with LY dye revealing a nerve bundle which is possibly the head retractor nerve (hrn). Scale bar: 250 μm.

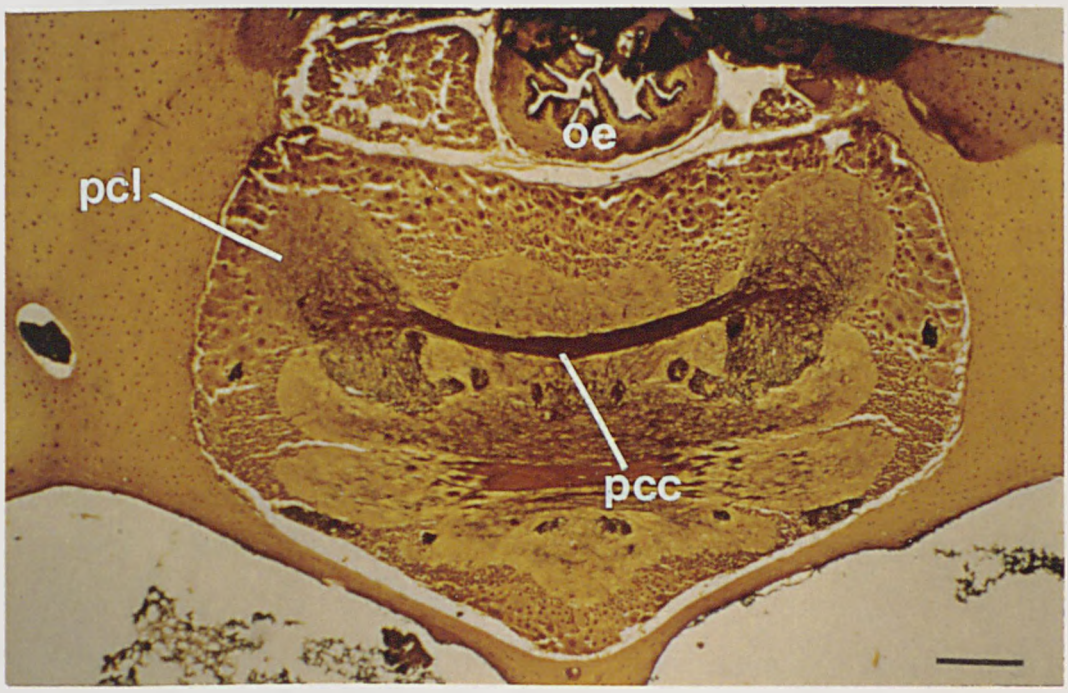
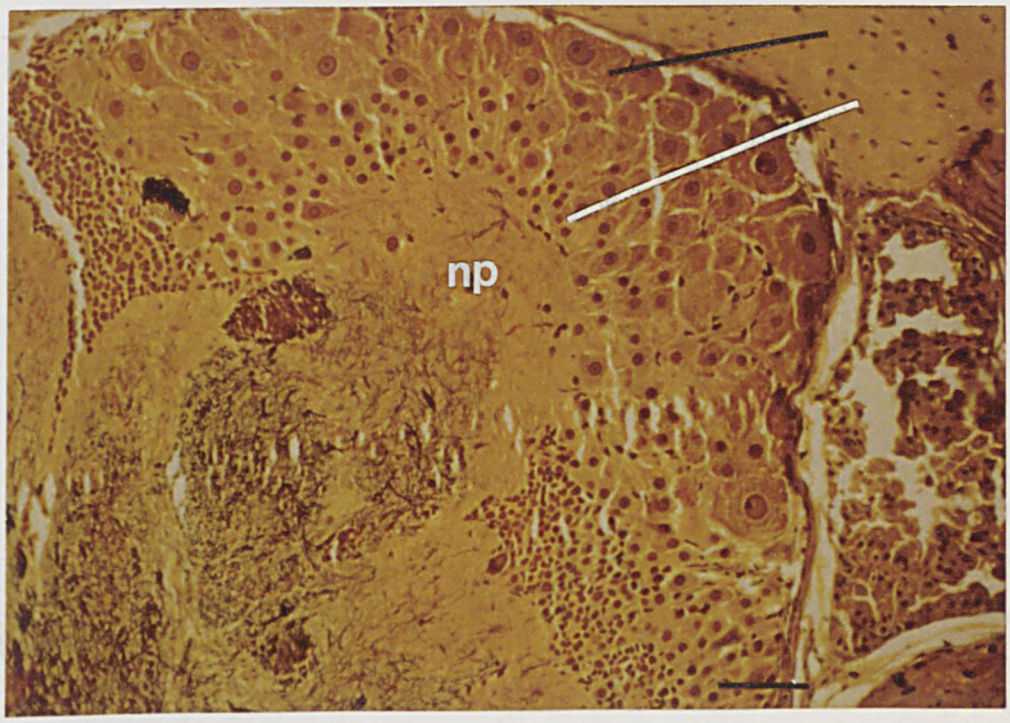


Fig 4.4 A silver stained transverse section of an *Alloteuthis* PCL showing the posterior chromatophore commissure (pcc) which unites the two PCLs. The oesophagus (oe) can be seen at the top of the picture. Scale bar: 500μm.

(a)



(b)

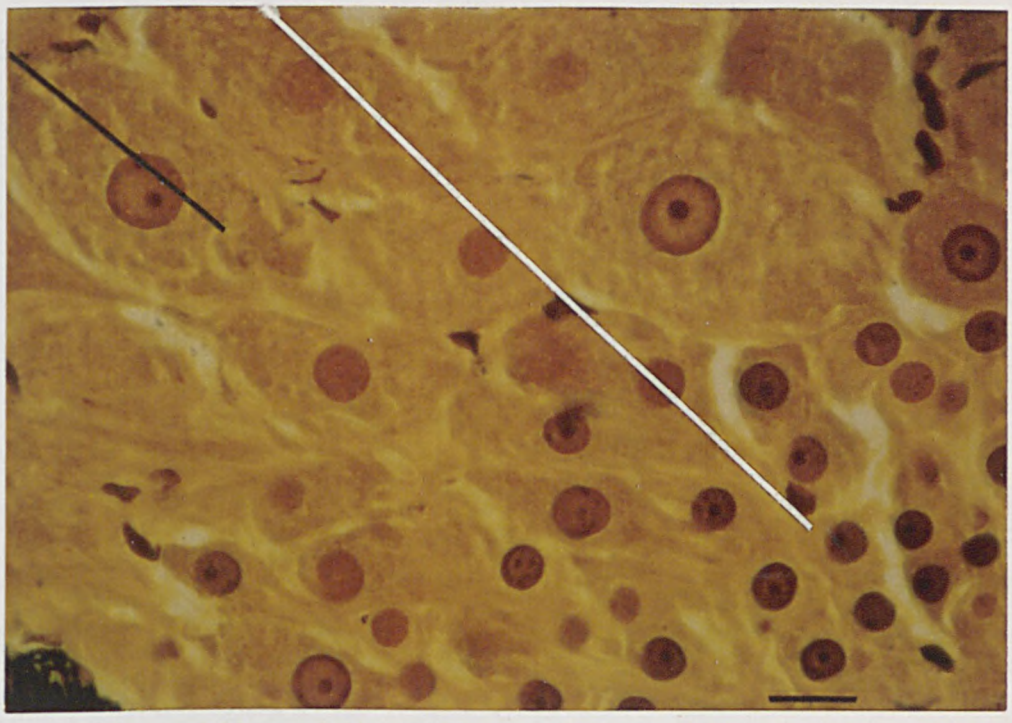


Fig 4.5 (a) A silver stained transverse section of an *Alloteuthis* PCL slice showing the gradation of cell sizes. Large cells occupy the outer part of the lobe (black line) and smaller cells (white line) occur towards the neuropil (np). Scale bar: 250 μm. (b) Higher power view of (a) showing the size difference between somata. Scale bar: 10 μm.

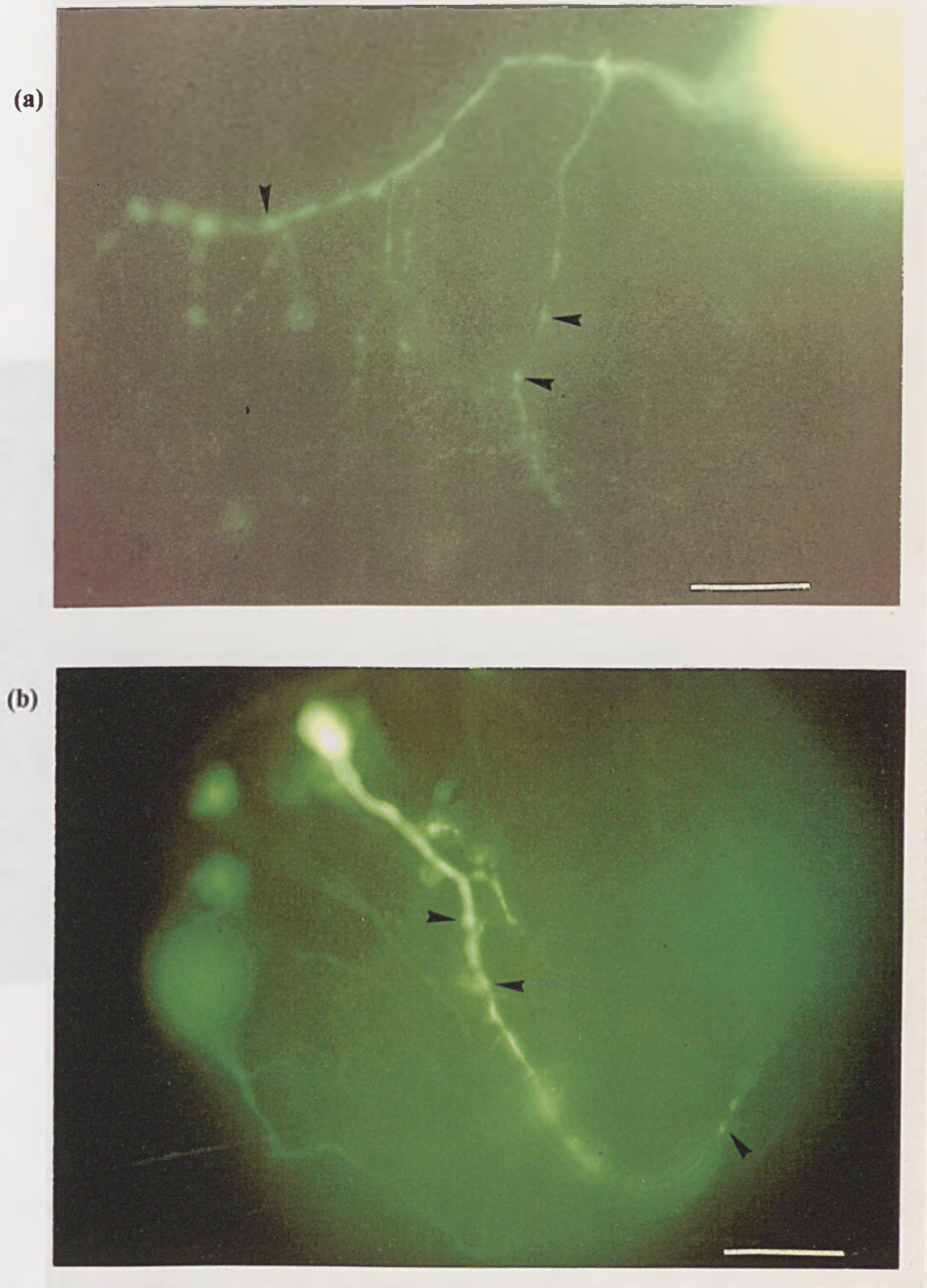


Fig 4.6 Lucifer yellow filled cells in the PCL of *Alloteuthis*. The dorsal side of the PCL is to the top of the photograph. Swellings (arrows) on the axon and dendrites of these cells may demonstrate dye-coupling to glial cells or sites of transmitter release (boutons). Scale bar: 10 μm (a), 40 μm (b).

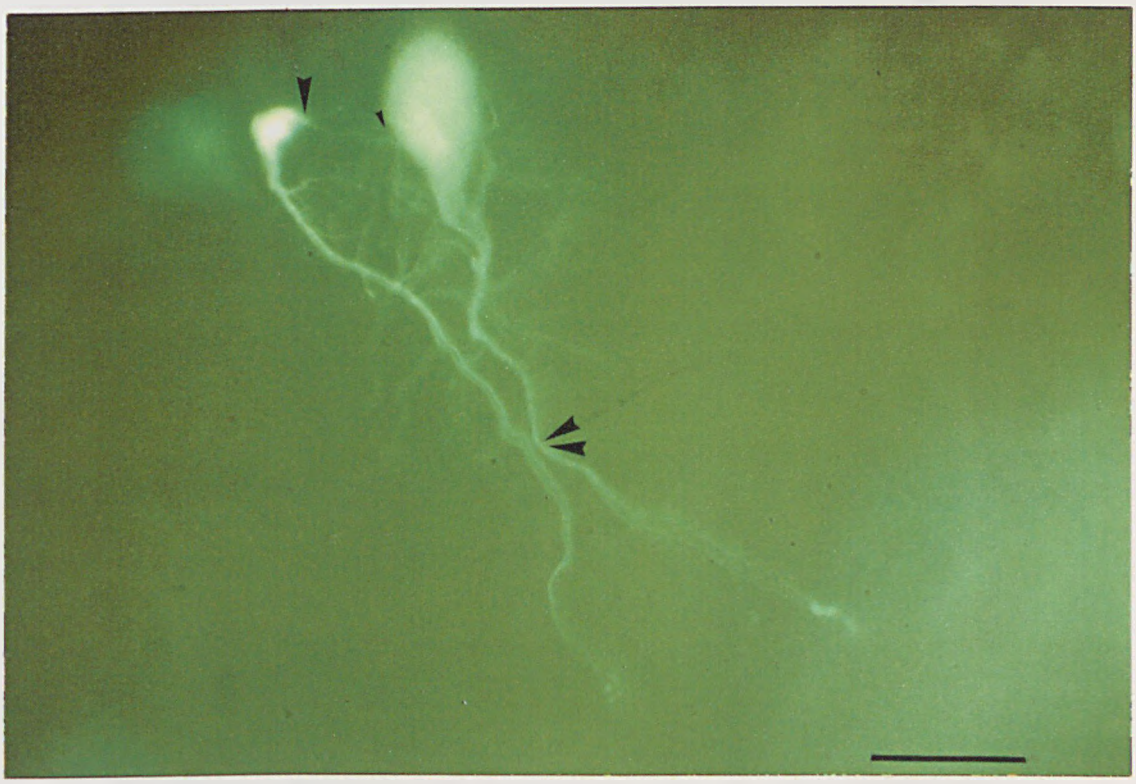


Fig 4.7 Two Lucifer yellow filled cells in the PCL of *Alloteuthis*. The dorsal side of the PCL is to the top of the photograph. The axons of the cells are in close association with each other (double arrow), and the dendrites of the two cells are intertwined. The cell on the left has a process on the soma (large arrow) which appears to make a connection with the other cell (small arrow). Scale bar: 40 μm.

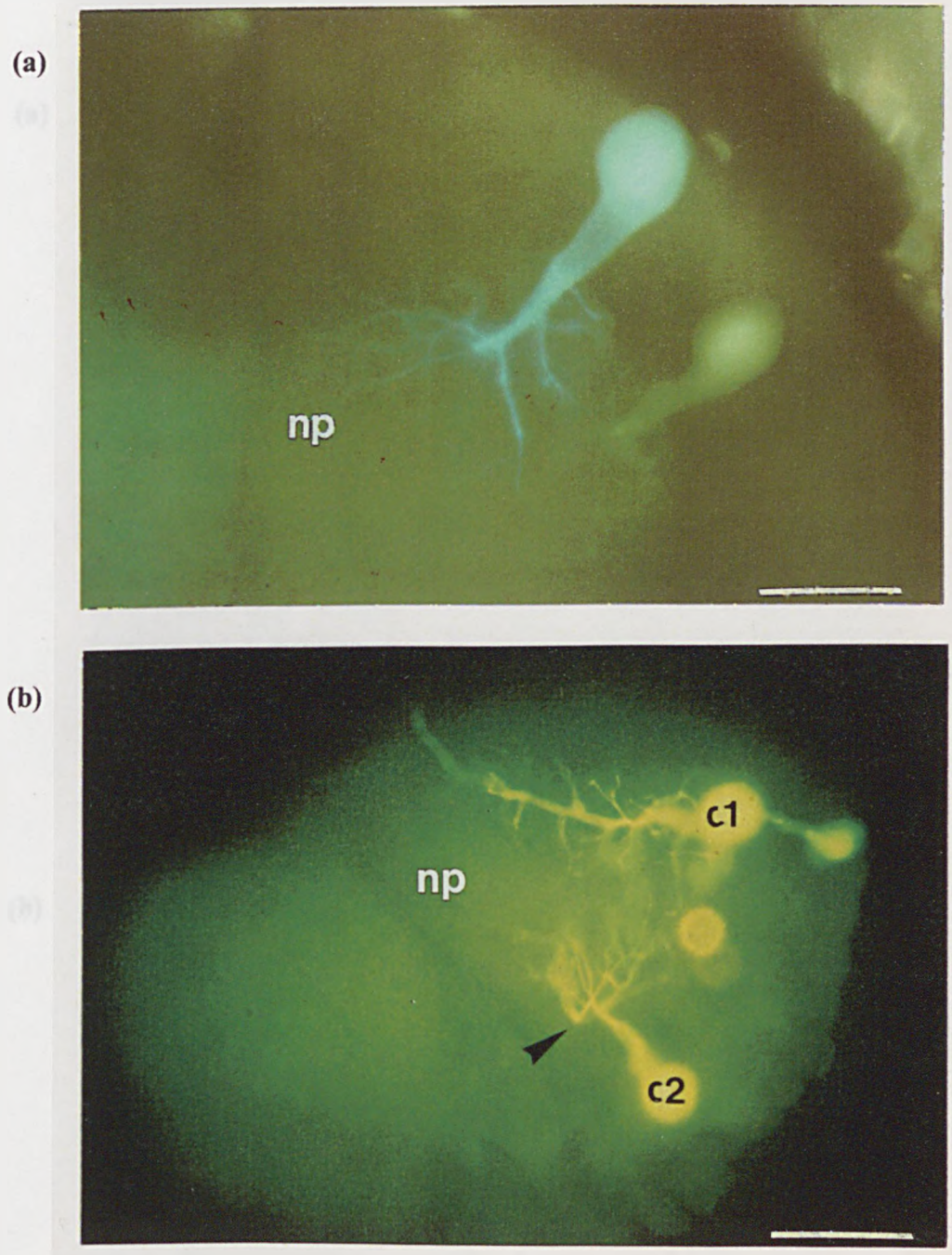
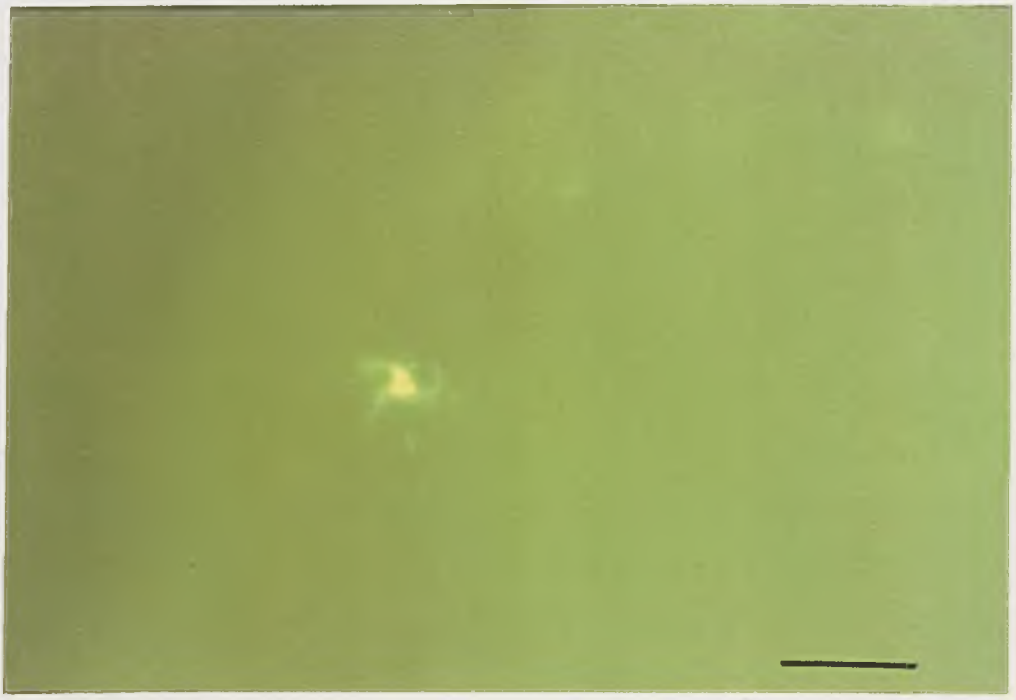


Fig 4.8 (a) An *Alloteuthis* PCL neuron stained by intracellular injection through the recording electrode with Cascade blue. This cell had no visible long axon travelling towards the PCL neuropil (np). Scale bar: 40μm. (b) Two morphologically very different dyed filled cells from an *Alloteuthis* slice. C1 resembled a motoneuron with a long axon that could be seen to travel into the pallial nerve. C2 had no long axon, and dendrites that were restricted to the neuropil of the PCL (arrow indicates the start of the neuropil) Scale bar: 40 μm.

(a)



(b)

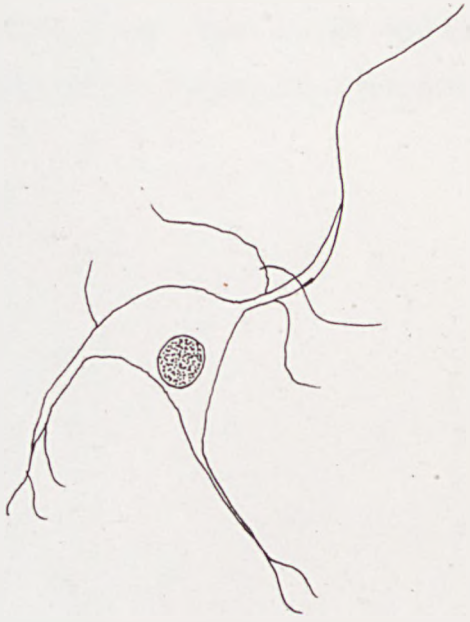
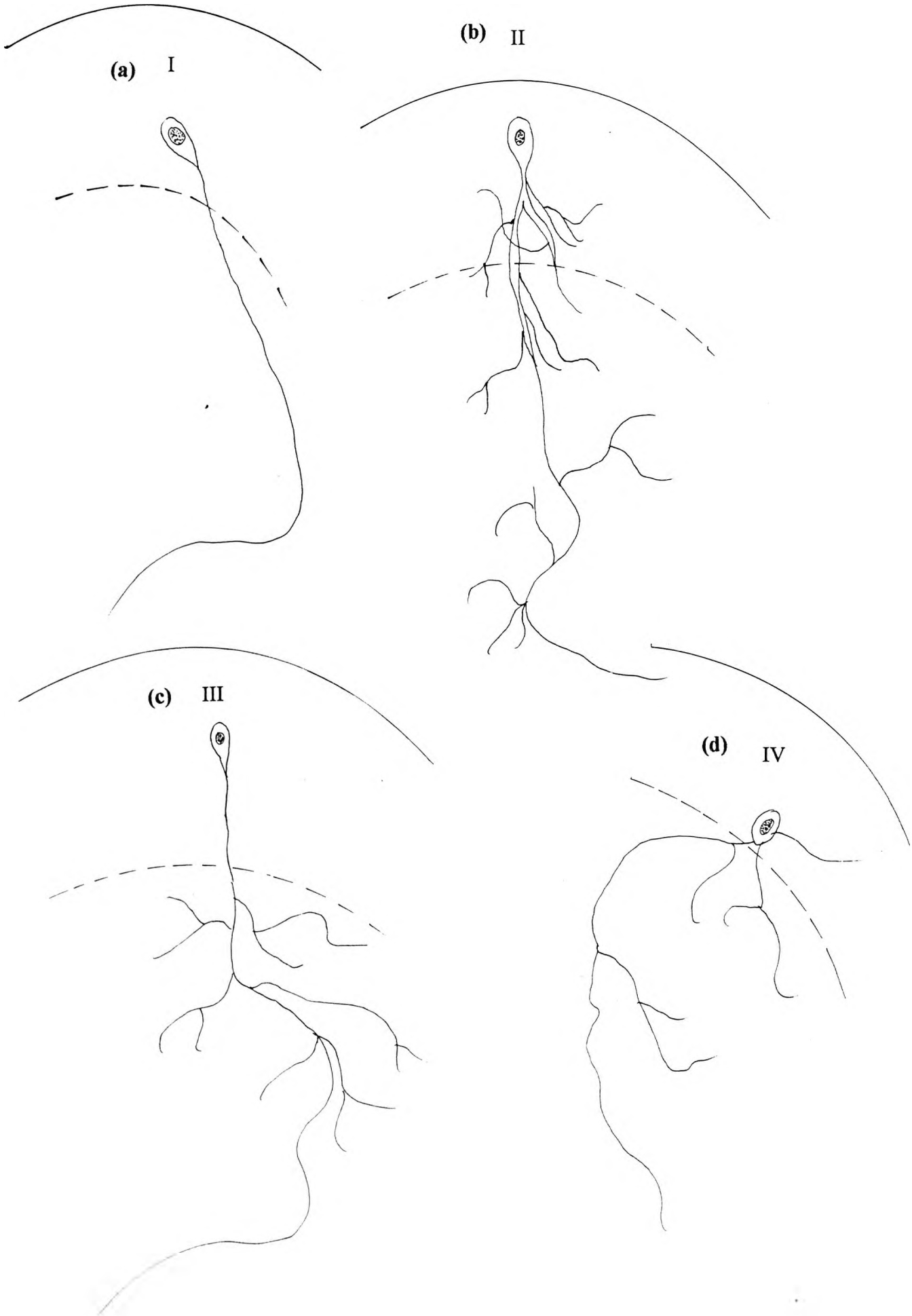


Fig 4.9 (a) An *Alloteuthis* PCL interneuron stained by intracellular injection through the recording electrode. Scale bar: 40 μm. (b) A drawing of the same cell. Note the triangular shape of the soma with dendrites protruding from its corners.

Fig 4.10 Traced drawings from a microscope image of Lucifer Yellow filled cells in the PCL of *Alloteuthis*. The dorsal side of the PCL is to the top in all cases. The solid line indicates the surface of the lobe/brain while the dotted line represents the boundary between the cell layer and the neuropil. (a) Type I cells had no arborizations on their axons and were found mainly in the inner cell layer. (b) Type II cells had an extensive array of arborizations that were not restricted to the neuropil, this type of cell was found predominantly in the outer cell layers of the PCL. (c) Type III cell arborizations were restricted to the neuropil, this cell was located mainly in the outer and middle cell layers. (d) Type IV cell types had two axons emanating from the cell soma, one always longer than the other. This type of cell was located only in the neuropil or inner cell layers. Scale bar: 40 μm.


(a) I
(b) II
(c) III
(d) IV

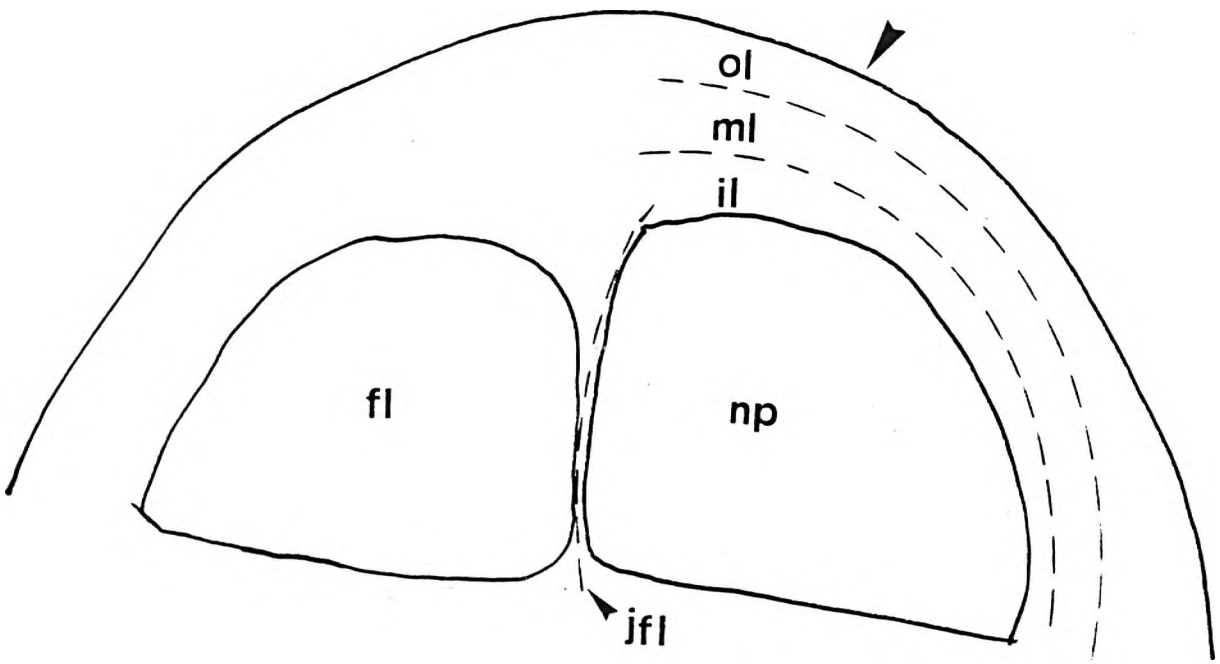
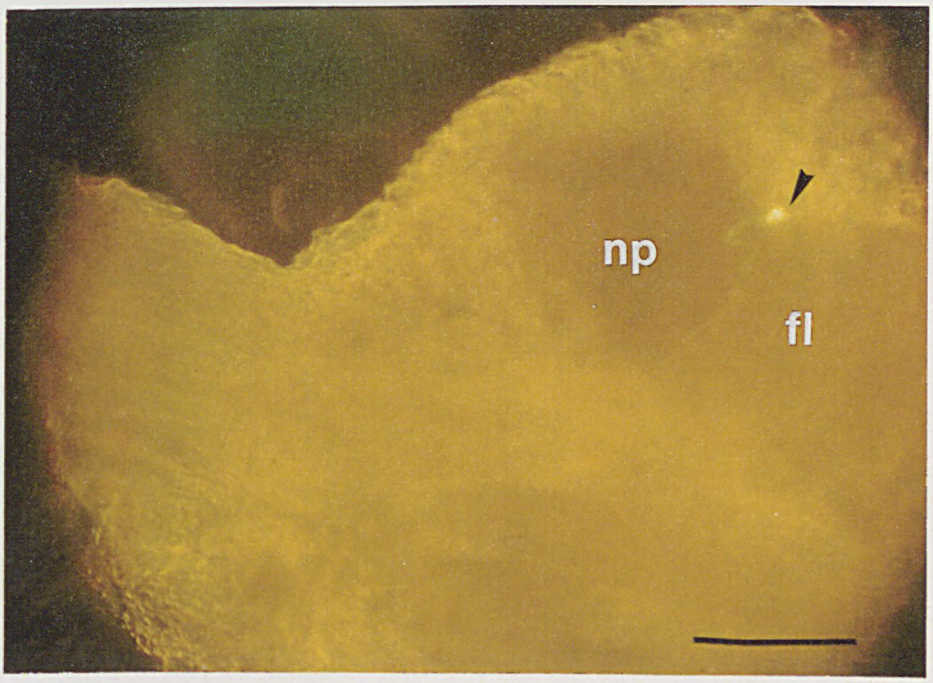


Fig 4.11 A schematic representation of the PCL of *Alloteuthis* with the cell layer of the lobe divided into outer cell layer (ol), mid cell layer (ml), inner cell layer (il). The junction between the fin lobe and PCL neuropils (jfl). The fin lobe neuropil (fl) and PCL neuropil (np) can be seen. The surface of the PCL is marked by an arrow head.

(a)



(b)

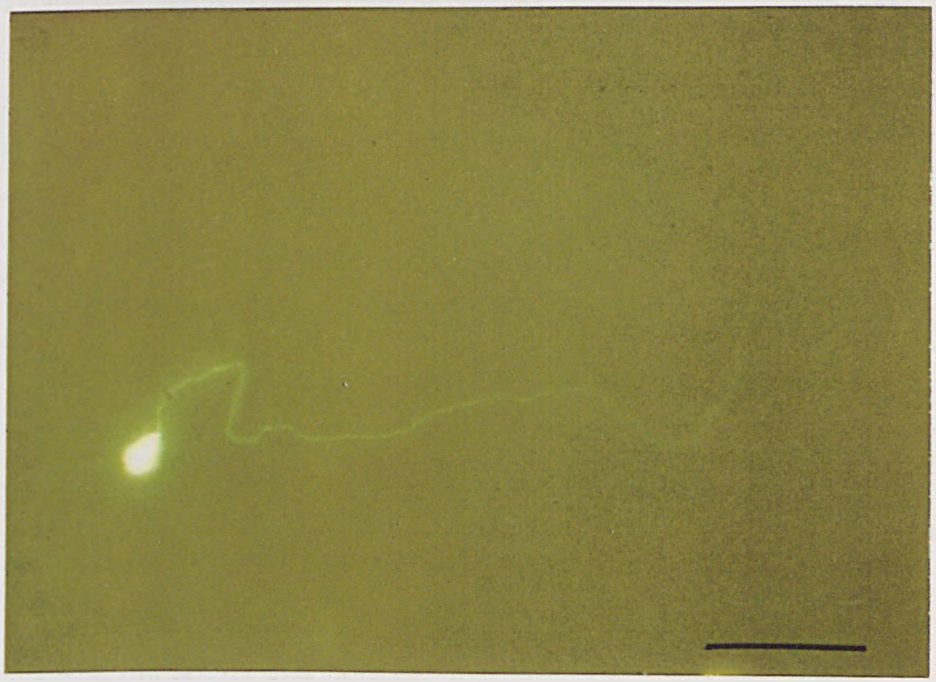


Fig 4.12 (a) A photograph demonstrating the location of an *Alloteuthis* PCL neuron stained through the recording electrode with Lucifer Yellow (arrow). This cell resembled a Type I cell which was situated at the junction of the fin lobe (fl) and PCL neuropil (np). Scale bar:250 μm. (b) Higher power view of (a) showing the long axon travelling towards the pallial nerve. No arborizations were visible. Scale bar: 40 μm.

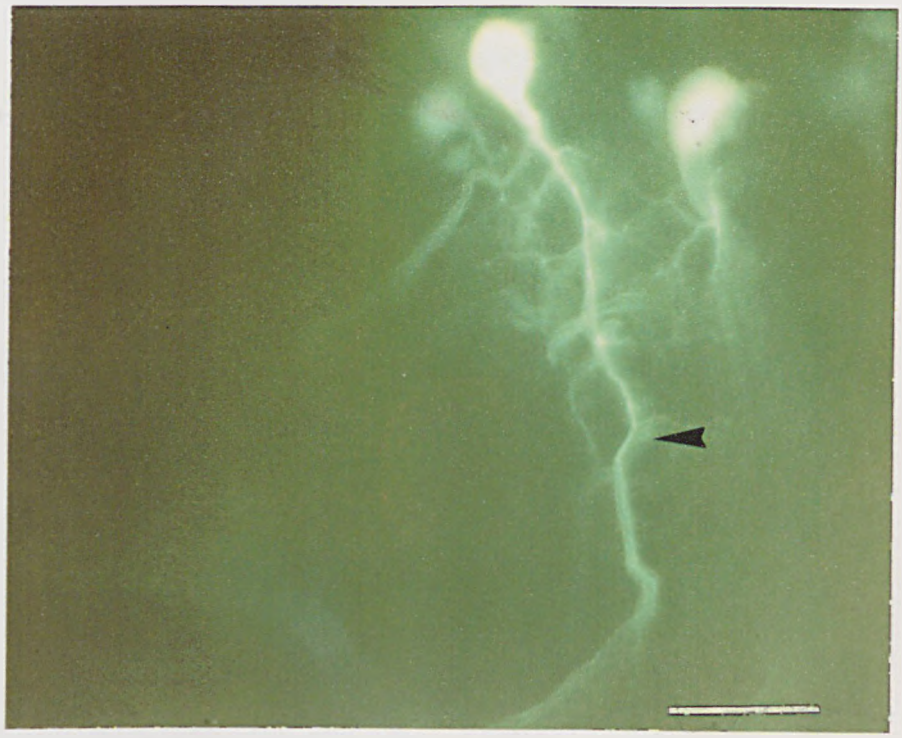


Fig 4.13 An example of a cell type II. The arborizations of this cell type are extensive, start near the cell soma, and are not restricted to the neuropil of the PCL (arrow indicates start of neuropil). Scale bar: 40 μm.

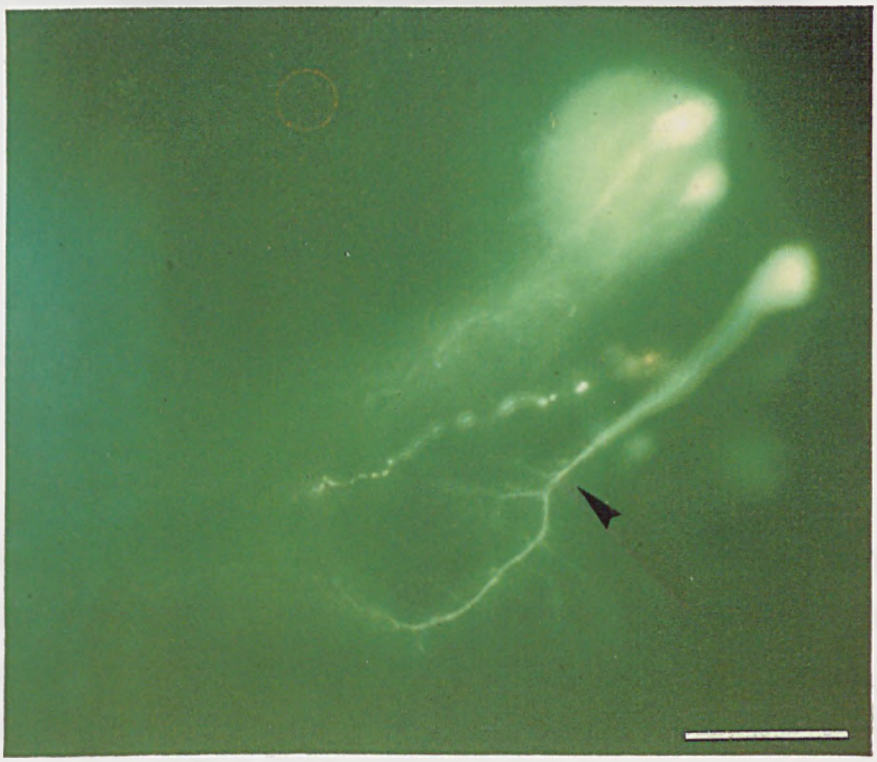
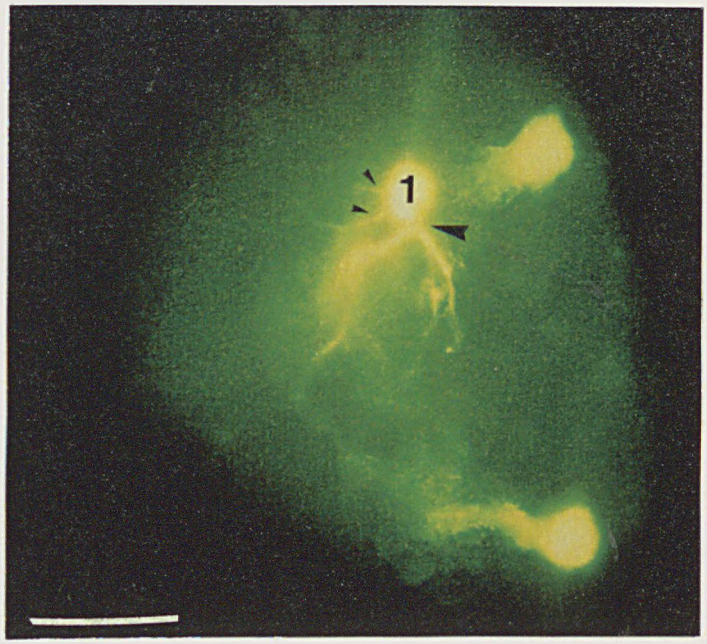


Fig 4.14 An *Alloteuthis* PCL neuron stained through the recording electrode with Lucifer Yellow. This cell resembled a Type III cell. The arborizations of this cell type started at some distance from the cell soma and were restricted to the neuropil (arrow indicates start of neuropil). Scale bar: 40 μm.

(a)



(b)

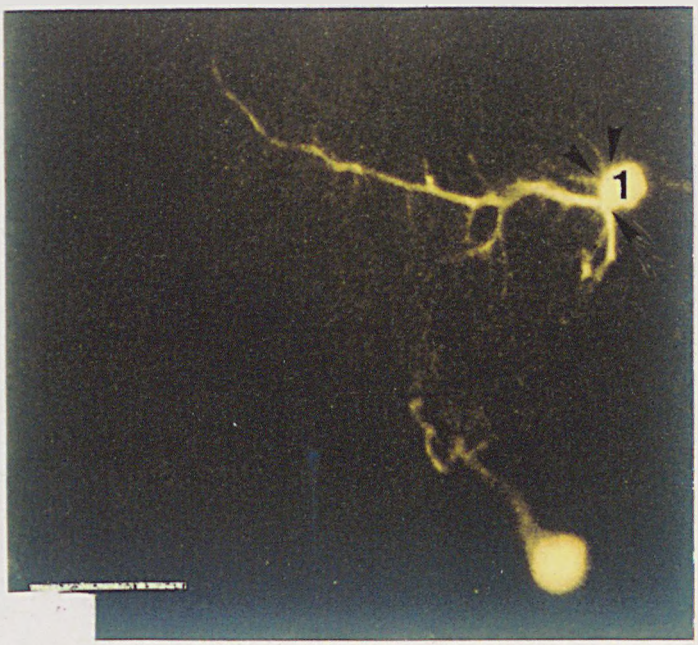


Fig 4.15 (a) *Alloteuthis* PCL neurons stained by intracellular injection through the recording electrode with Lucifer Yellow. Cell 1 was of a Type IV cell. The cell has a soma dendrite or axon which is as thick as the main axon emanating from the cell soma (large arrow). Finer soma dendrites can also be seen (small arrows). This cell is the same as that seen in Figure 4.8 and was located in the inner cell layers. Scale bar: 40 μm (b) The same cell was scanned at 10μm sections using a confocal microscope, this revealed the cell in its entirety including the soma dendrites (arrows). Scale bar: 40 μm.

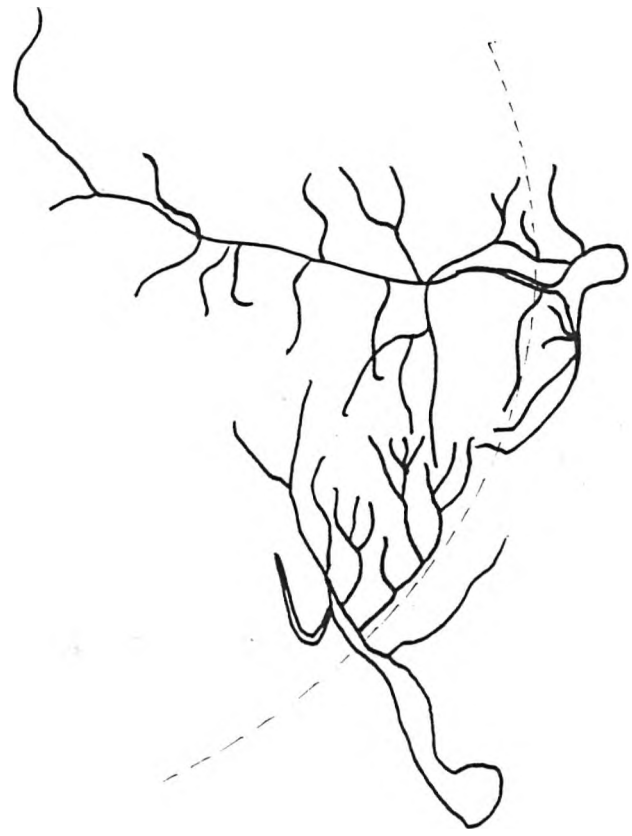


Fig 4.16 Camera lucida drawings of the same cells as in figure 4.8 & 4.15. These revealed the close association of cell dendrites and the differences in their anatomy. The dendrites of both the cells started at the beginning of the PCL neuropil (dashed line). Scale bar: 40 μm.

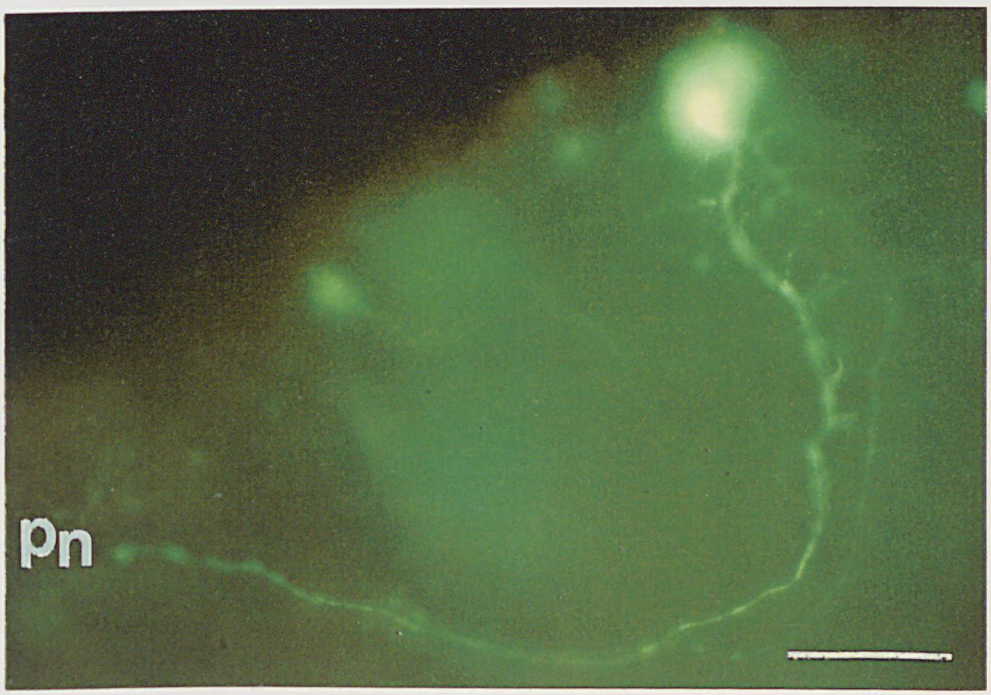


Fig 4.17 Lucifer Yellow filled cells in the PCL of *Alloteuthis*. These two cells are dye-coupled. The axons of the two cells can be seen travelling towards the pallial nerve (pn). Scale bar: 40 μm.

Table 4.1. Distribution of the four cell types in the PCL

Cell type	Total No. of cells	Neuropil		Junction with the fin lobe neuropil		Inner cell layer		Middle cell layer		Outer cell layer	
		No.	%	No.	%	No.	%	No.	%	No.	%
I	10	-	-	2	20	5	50	2	20	1	10
II	50	-	-	-	-	13	26	15	30	22	44
III	33	-	-	-	-	-	-	12	36	21	64
IV	3	1	33	1	33	1	33	-	-	-	-
Total No.	96	1		3		19		29		44	

Table 4.2 Mean number of dendrites per cell at the different locations within the PCL.

Location of cell soma	Total No. of cells	Number of dendrites per cell
PCL Neuropil	1	10
Junction with the fin lobe neuropil	3	2 ± 1
Inner cell layer	19	5 ± 2
Middle cell layer	29	12 ± 4
Outer cell layer	44	15 ± 5

Values are in means ± SD.

CHAPTER FIVE

INTRACELLULAR AND EXTRACELLULAR INVESTIGATION OF EVOKED RESPONSES IN PCL NEURONS GENERATED BY PALLIAL NERVE STIMULATION

5.1 Introduction

Despite various studies on the anatomy of the cephalopod chromatophore system (e.g. Sereni & Young, 1932; Dubas et al, 1986a) showing that axons from PCL neurons travel via the ipsilateral pallial nerve, through the ipsilateral stellate ganglion, probably without synapsing, to the mantle skin where they innervate chromatophore muscle fibres, there has been very little physiological work on the system. Boycott (1961) was the first to show that direct electrical stimulation of the PCL region in Sepia caused changes in chromatophore activity, thus demonstrating physiologically that the PCL is involved in the control of the mantle chromatophores. This was later supported by the results of Chichery & Chanelet (1976), also in cuttlefish, and extended to squid by Dubas et al (1986b) who made a careful study of the likely numbers of chromatophores innervated by single or groups of PCL motoneurons and their spatial relationships with regard to the position of the chromatophores on the body and the position of the neuron soma in the brain

Thus there has been some progress in understanding the physiology of the peripheral system, but what about the central system and its control of the chromatophore patterning ? Although gross field potential recordings could be made from the intact cephalopod brain (Bullock, 1984; Bleckmann et al, 1991) this could not be well correlated with chromatophore activity. Recently however, direct recordings from the PCL within the brain were achieved by Miyan & Messenger (1995) using an immobilised octopus preparation. They showed that intracellular recordings could be obtained from unidentified PCL cells. Although a valuable step forward, this meant that cells were mainly recorded from the periphery of the lobe. Also this type of preparation would be difficult to exploit further in the UK because of the current legal status of octopus and the likely extension of this protection to other cephalopods. An alternative approach has

therefore been developed which uses slices of the squid brain, cut so that they contain at least a portion of the PCL and the ipsilateral pallial nerve, for physiological investigation. The previous Chapter revealed the gross anatomy of the PCL and the detailed morphologies of individual PCL neurons. The aim of this section is to demonstrate that electrical activity can be recorded from brain slices containing the PCL and that, if the pallial nerve is activated by direct electrical stimulation, then the subsequent activity within the slice is well correlated with the arrangement of PCL neurons. In addition, it is shown that pallial nerve stimulation can be used to identify individual cells in the PCL as chromatophore muscle motoneuron.

The results are discussed in terms of: 1) the viability of the PCL brain slice preparation, 2) the identification of chromatophore motoneurons within the PCL, and 3) the influence of the pallial nerve on the PCL activity.

5.2 Results

5.2.1. Characteristics of antidromic responses

Electrical recordings from PCL cells were identified as being intracellular if a cell membrane resting potential in excess of -20mV could be obtained. In many cases, cell action potentials or excitatory postsynaptic potentials (EPSPs) in the PCL neuron were observed immediately after cell penetration. Subsequent to the electrical recordings of the cell's activity, many cells were injected with a fluorescent dye through the microelectrode. This provided a convenient way of confirming that the cell penetrated was a motoneuron, and also that the recording electrode was indeed intracellular, as extracellular injection of dye would not have stained the cells.

Electrical stimulation of the pallial nerve evoked a depolarising, spike like, potential, of up to 30 mV in amplitude (Fig.5.1). The response was all or nothing, with a distinct stimulus threshold, at which the response appeared. In different cells, the responses showed some degree of variation in the amplitude and rise time of the evoked spike (Fig. 5.1). This variation in amplitude and spike rise time is likely to be due to the location of the intracellular electrode within the

neuron. For example, an electrode inserted into the cell axon produced a sharp response with a large amplitude (Fig. 5.1a). Whereas, when the recording was made from the cell soma, where the action potential was not actively propagated, the potential was likely to be smaller and have a longer rise time (Fig. 5.1b). The latency of the response recorded in different cells increased the further away that cell was from the stimulating electrodes on the pallial nerve. The potential recorded from a cell had a constant latency for repeated stimuli and was of the same amplitude and shape as the action potentials evoked by intracellular current injection (see chapter 6). The potential did not increase in amplitude with increasing stimulus amplitude. Thus, it is likely to be an antidromic action potential arising from activation of the cell's axon in the pallial nerve, and identifies the cell as a chromatophore motoneuron. Therefore antidromic potentials were characterised by the following criteria; 1) constant latency; 2) no increase in amplitude with increasing stimulus; 3) similar amplitude and shape to intracellular action potentials. The small amplitude of the antidromic spikes implies that the squid cell soma does not carry a regenerative action potential but that the axon spike is conducted electronically to the cell soma. This has also been previously reported for cephalopod central (Laverack, 1980) and peripheral (Williamson, 1989) neurons. Since the cell soma lie outside the neuropil area, the activity seen by the microelectrodes will be greatly attenuated and represent only a fraction of the total activity.

5.2.2. Field potentials

Extracellular field potential recordings are a convenient and effective way of rapidly assessing and monitoring the activity of a neuronal population. They are generated by the activity of groups of cells and are a reflection of what is happening inside the cell. The field potential produced by a group of cells reflects in an indirect way the membrane potential changes such as action potentials and synaptic potentials. A study of such potentials therefore gives invaluable information regarding the average activity of a group of cells, and is a basic prerequisite for understanding the physiological characteristics of a neural assembly.

Field potentials were used in this investigation to map the route of the pallial nerve fibres into, and within, the PCL. They were also used to identify areas in the PCL that could be associated with motoneuron activity, and for determining the location of synaptic contact within the lobe through the localisation of complex synaptic fields.

Excitatory fibres in the PCL were stimulated and the evoked potentials were recorded thoughout the PCL. The potentials were amplified and displayed on an oscilloscope whose amplifiers sent the signal to a Cambridge Electronic Design 1401 intelligent interface, controlled by a CED 16 channel signal averager programme and a PC 386 computer. The initial component of the response was the presynaptic fibre spike, which occurred due to the action potentials in the presynaptic fibres. This was a compound action potential recorded from axons and nerve terminals and reflects currents generated by the synchronous activation of large numbers of neurons. The amplitude of the response was proportional to the number of activated neurons.

The first finding was that the field potentials varied in amplitude and shape from locus to locus (Fig. 5.2). Figure 5.2 illustrates the response from the recording electrode elicited by pallial nerve stimulation at pulses of one per second, recorded in the PCL. Localisation of the position of the extracellular electrode tip after each recording session was achieved only grossly, with the position of the response being marked on a grid placed over a drawing of the slice. After the recording session, the same slice had the pallial nerve dye-filled by retrograde transport of Lucifer Yellow, to reveal the morphology of the pallial nerve fibres. This revealed the close correlation between the evoked potential recorded and the pallial nerve fibres. The field potential traces were superimposed upon the same dye-filled slice (Fig. 5.2).

The shape and distribution of the evoked field potential provides information on the distribution of neurons and their contacts within the PCL. The most obvious extracellular potentials observed were the large compound action potentials, resulting from the synchronous discharge of many axons, seen by placing the recording electrode on, or close to, the pallial nerve (Fig. 5.2). Extracellular field potentials recorded from the neuropil of the PCL indicated the

position of motoneurons activated by pallial nerve stimulation. Some loci showed an evoked response on a signal sweep, without averaging, while some loci showed evoked responses only after averaging. Others showed no evoked potentials even with several hundred sweeps. The responses recorded from the periphery of the lobe only became apparent sometimes after several hundred sweeps, while those taken from the neuropil and near the pallial nerve often required no averaging and the response could be seen on a single sweep. This could possibly be due to current spread from the stimulating electrode or dispersion of the pallial nerve fibres, seen in Figure 5.2, making the response weaker. Therefore a major factor in determining whether evoked potentials were viable was the amplitude of ongoing activity at that time and place; this varies, as noted in figure 5.2.

Two characteristics of the evoked field potentials are particularly worthy of note:

1) The largest and earliest potentials are present on the pallial nerve.

2) The smallest and latest potentials are present in the periphery of the lobe.

Therefore, the amplitude and latency of the response depends somewhat on the stimulus intensity, duration, and distance of the loci from the stimulating electrode. Small responses, requiring the averaging of several hundred sweeps, are quite local, being seen only in a few loci in the periphery of the lobe. This means that we cannot be sure that we have encountered the best loci.

In studying evoked field potentials in the brain, one of the most common problems encountered was the analysis of fields generated by the synchronous activation of large numbers of cells belonging to a certain pool or nucleus. The evoked field responses seen in the squid (Fig 5.2) are similar to an open field neuron pool (Stephen 1974). Here the neural elements are orientated so that the somata are all in one region and the dendrites are orientated towards the opposite end. This produces a characteristic open field with different amplitudes of the evoked response, arranged in a similar way to those fields seen in figure 5.2. This arrangement is that typically found in the cerebral cortex, the cerebellum, or hippocampus (Stephen, 1974). If action potentials invade the dendrite of the neurons in the pool, an electrode located midway between the soma and dendrites

will record a positive-negative-positive field as the action potential approaches, reaches and leaves the recording site. An example of this field can be seen in responses located near the pallial nerve in figure 5.2.

In order to understand the meaning of the field potentials generated by the activation of any neural assembly, a thorough knowledge of the microscopic organisation of the neural elements in question is essential. Therefore the information obtained by dye filling experiments here and in Chapter 4 have assisted in the analysis of the field potentials in this chapter and in further chapters.

The way in which field potential data is displayed can assist in the interpretation and presentation of the field potentials. An alternative way of representing the field potential data is shown in Figure 5.3. This shows a contour plot, which to our knowledge has not been produced before. Each field potential shown in figure 5.2 had its position marked by placing a grid over the photograph of the slice and the position of the field was measured in relation to its distance form the stimulating electrodes on the pallial nerve and the distance from the top of the lobe. The amplitude of the potential at each location, from Fig 5.2, was measured and plotted against the measurements discussed above. This produced a 3-dimensional representation of the fields evoked thoughout the slice (Fig 5.3). This contour surface plot clearly shows the large field caused by the pallial nerve axons as they enter the slice and spread throughout the PCL neuropil. The contour plot reveals, not only the accurate location and distribution of pallial nerve axons within the slice, but also local areas of high PCL motoneuron activity could be further investigated. These areas could be due to a concentration of active dendrites or perhaps clusters of cell bodies. They are clearly areas of high priority for further investigation using intracellular electrodes. Field potentials, are therefore, a physiological sign of brain activity which can be explained in terms of the anatomy and electrical activity of the PCL motoneurons, evoked by the pallial nerve.

The evoked field potential recordings found in the squid, represented in this study, are similar to those reported for *Octopus*, which is distinct from other invertebrates and is in general more vertebrate-like (Bullock, 1984).

5.3 Summary

1) The brain slice has proved a viable preparation for studying chromatophore motoneurons, as axons remain intact for stimulation and retrograde dye transport filling of the pallial nerve.

2) With the technique described it was possible to record, for the first time, evoked antidromic action potentials from individual PCL cells of *Alloteuthis* and *Loligo*, thereby identifying them as chromatophore motoneurons.

3) The latencies and amplitudes of the evoked antidromic action potentials vary in different cells according to the location of the chromatophore motoneuron within the PCL.

4) The brain slice is also viable for studying evoked extracellular field potentials in the squid brain, previously unstudied. The responses are comparable to those found for *Octopus* by other workers (Bullock, 1984).

5) The field potentials varied in size and shape from locus to locus. A contour plot was produced which showed that a large field is produced by the pallial nerves as they enter the slice and spread through the neuropil.

6) The distribution and amplitude of evoked field potentials agrees in part with the known anatomy of the PCL and the distribution of the pallial nerve fibres thoughout the PCL. Areas of high electrically activity have thus been identified.

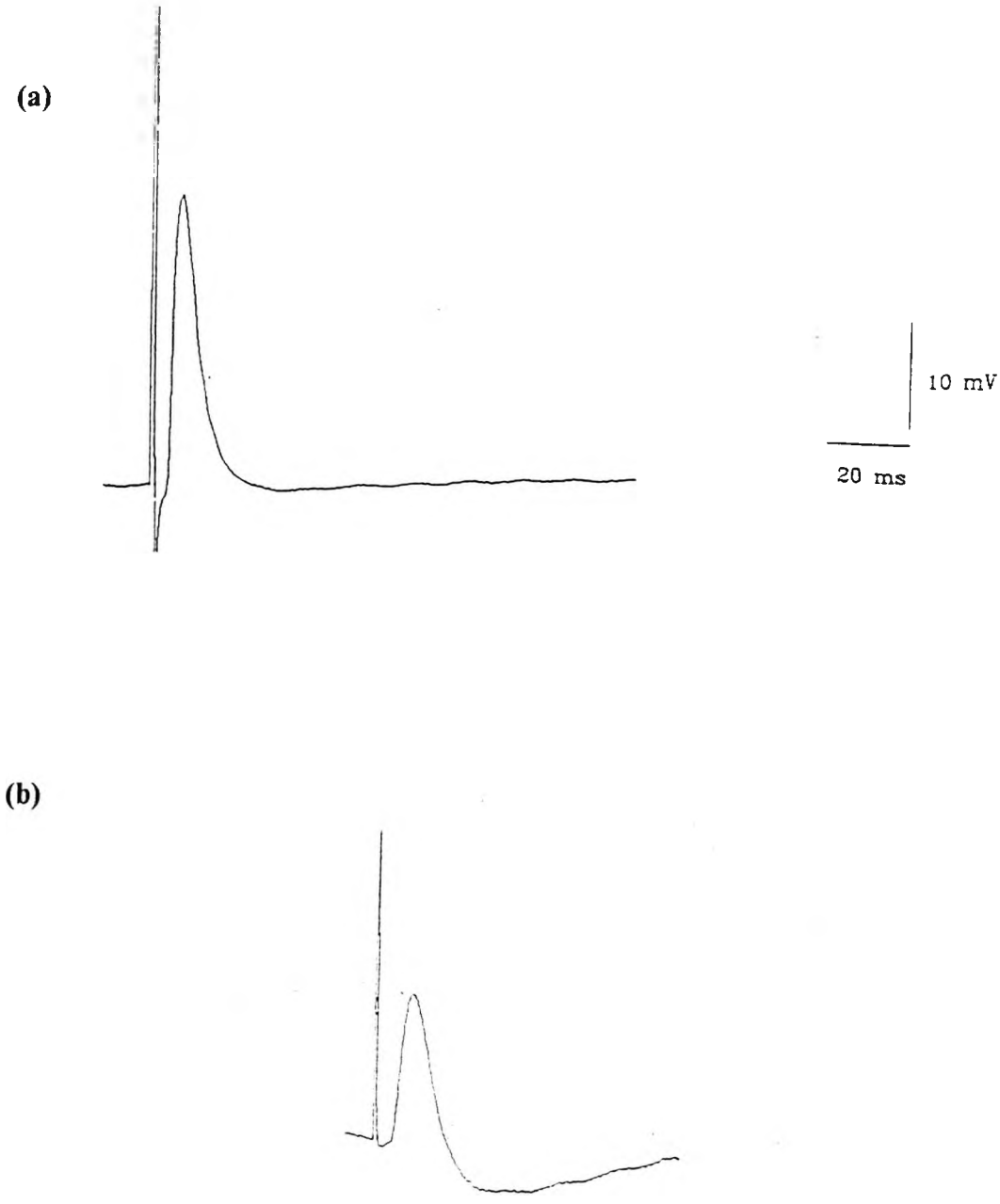
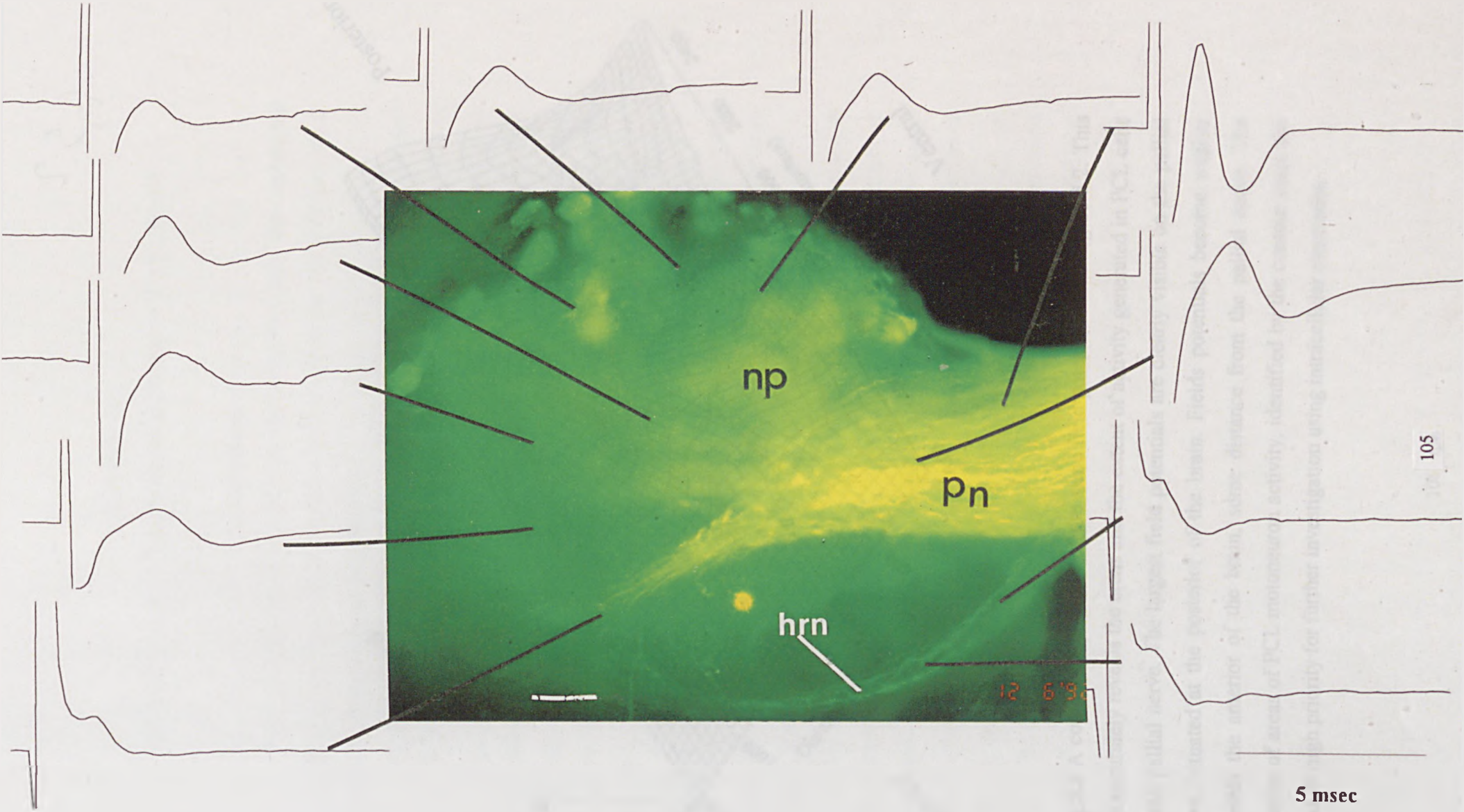


Fig 5.1 Evoked antidromic action potentials recorded from two separate *Alloteuthis subulata* PCL motoneurons generated by electrical stimulation of the pallial nerve. (a) This recording was probably taken from the cell axon, resulting in a large short duration response. (b) This recording was probably taken from the cell soma, resulting in a smaller, broader, response.

Fig 5.2 Evoked extracellular field potentials, generated by electrical stimulation of the pallial nerve, in a PCL slice from *Alloteuthis subulata*. Field potentials were taken from the positions shown (Black lines), subsequently the pallial nerve (pn) was backed filled with Lucifer Yellow dye. Large compound action potentials were recorded from the pallial nerve. Smaller field potentials were recorded from the head retractor nerve (hrn) and the PCL neuropil (np). Scale bar: 250 μm.


np
pn
hrn
5 msec

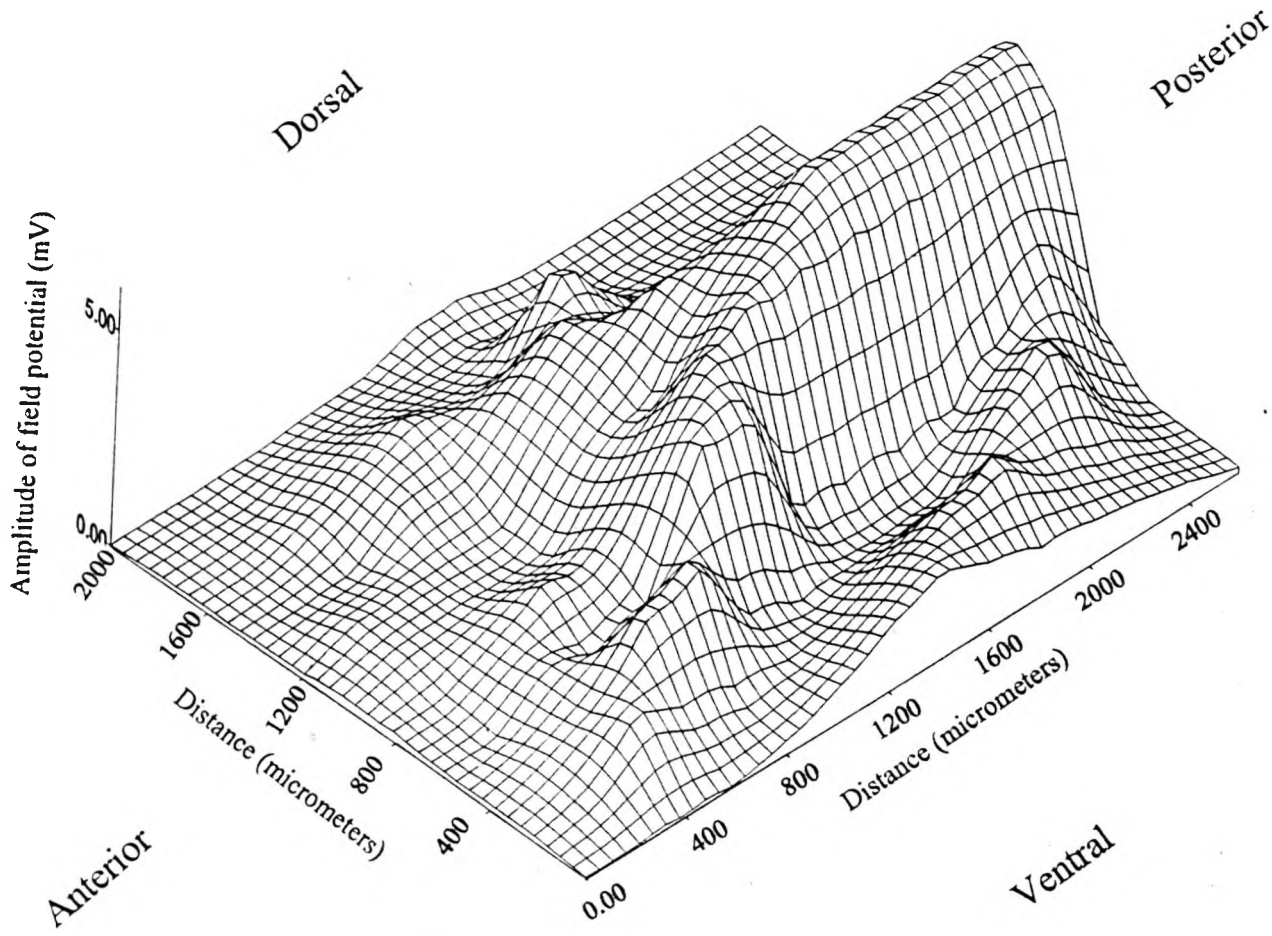


Fig 5.3 A contour surface plot of the same field potentials as in figure 5.2. This plot accurately reveals the areas and the extent of activity generated in PCL cells by the pallial nerve. The largest field potentials are clearly visible on the pallial nerve, situated at the posterior of the brain. Fields potentials become weaker towards the anterior of the brain, some distance from the pallial nerve. The location of areas of PCL motoneuron activity, identified by the contour map, are areas of high priority for further investigation using intracellular electrodes.

CHAPTER SIX

INTRACELLULAR RECORDINGS FROM PCL NEURONS

6.1 Introduction

There is a significant lack of electrical recordings from the cephalopod brain due to the difficulties associated with immobilisation and poor survival of animals when anaesthetised or perfused.

It was Laverack (1980) who recorded the first activity from cells in brain of the cephalopod, however, the locations of the recordings were not identified and he was unable to reach cells deep within the brain. Miyan & Messenger (1995) used a whole brain preparation to record for the first time intracellular recordings from the posterior chromatophore lobe (PCL) of the octopus. However, cells were only recorded from the periphery of the PCL, no correlation was made between electrical activity and morphology of the cells, and chromatophore motoneurons were not identified.

It is clear, therefore, that we still lack some understanding of the electrical activity of PCL cells and their corresponding morphology, as well as the activity of cells located in the inner lobe regions near the neuropil.

The aim of this part of the study, therefore, was to characterise the electrical properties of neurons within the PCL, from the periphery of the lobe to the neuropil, and correlate this activity to cell morphology. The purpose being to identify any differences in the physiology of the four morphological sub-types of PCL cells identified in Chapter Four.

The findings presented here show the first intracellular recordings taken from identified neurons in the PCL of *Alloteuthis subulata*, and provide the first report of the brain slice preparation being used to record activity from the PCL (for full methods see section 2.4).

6.2 Results

The results are based on a total of 503 recordings from individual cells within the PCL; these recordings were obtained from 146 different PCL slice preparations. The majority of the recordings made were from the squid, *Alloteuthis subulata* and *Loligo vulgaris*, with a few being made from *Eledone cirrhosa* and *Octopus vulgaris* PCL cells (Table 6.1). 115 cells from the PCL were successfully filled with the dye Lucifer Yellow. The majority of the dye filled cells were obtained from the squid *Alloteuthis subulata*; these cells could be subdivided into four morphological cell types on the basis of differences in the dendrite positions on the cell axon (Chapter 4) (Table 6.1). Cell type II was the most common cell type stained (Table 6.1). Cells were identified as chromatophore motoneurons by electrically stimulating the pallial nerve to evoke an antidromic spike (Chapter 5). The identity of each cell as a motoneuron could be further confirmed by observing the dye filled axon as it left the brain via the pallial nerve. The criteria used for accepting a penetration as intracellular, were that there was a sudden change in the microelectrode potential to a sustained membrane resting potential (MRP) of more than –20mV. Clearly a MRP of –20mV was the lowest acceptable value and indicated that the cell and/or the slice was in poor condition but that recordings were still possible.

6.2.1 Membrane properties of PCL cells.

The intracellular recordings revealed that the chromatophore cells in the PCL of *Alloteuthis subulata* have a mean membrane resting potential (MRP) of –37.5 ± 8.4 mV (± SD, n=266), with a large range from –20 to –60 mV. *Loligo vulgaris* had a mean MRP of –40.9 ± 11.0 mV (± SD, n=162), with a range of –23 to –62. *Eledone cirrhosa* had a mean MRP of –23.8. ± 13.5 mV (± SD, n=42), with a range of –20 to –56. *Octopus vulgaris* had a mean MRP of –32.1 ± 11.4 mV (± SD, n=33), with a range of –22 to –59 (Table 6.2). Therefore, between *Alloteuthis subulata*, *Loligo vulgaris*, *Eledone cirrhosa* and *Octopus vulgaris* the MRPs varied, with their means differing with a high significance ($P < 0.001$,

Student's *t* test). The biggest difference in membrane potential was between *Loligo* and *Eledone* and the smallest between *Eledone* and *Octopus*. This variation could be an indication of the cell's viability in the brain slice rather than real differences in the MRPs among the different cephalopod species.

The cell resting potential did not change significantly for a given species over the period of the project ((Fig. 6.1a), despite the changes in the ASW solutions and improvements in both the slicing and cell penetration techniques. There was, however, some indication of a seasonal variation in the MRPs of the PCL cells (Fig. 6.1a). Consecutively over the years of 1992 and 1993, there appeared to be a decrease in the MRP of the cells during the winter months of February, March and April, a steady increase in the MRP during the summer months of June, July, August, and September, and a steady decrease again during the winter months of October, November, and December (Fig. 6.1a). This phenomenon could be due to changes in the sea temperature, the condition of the animals during the winter months due to adverse weather conditions, and the fact that less animals were caught during the winter. The distribution of MRPs was approximately normal over the period of the project (Fig. 6.1b) with a mean value of -37.5mV. This value is consistent with that found in other cephalopod brain studies (Williamson & Budelmann, 1991; Miyan & Messenger, 1995)

There was a variation in the MRP among the cells in the same slice; with the MRP ranging from -20mV to -60mV (n=266). This range is also consistent with the ranges shown by other workers (Williamson & Budelmann, 1991; Miyan & Messenger, 1995). This large variation in MRP values may indicate a difference in the quality of cells rather than a large difference in the MRP itself. For example, smaller cells may be subject to more damage during intracellular impalements than larger ones. The depth at which the cell lies might also affect the MRP; cells at the surface may be more prone to damage to their dendrites during slicing, but less susceptible to oxygen depletion compared to those deeper within the slice.

6.2.2 Membrane properties of the different morphological cell types of the PCL in Alloteuthis subulata.

Since the majority of intracellular recordings and dye fills were obtained from *Alloteuthis*, comparisons were made between the cell sub-types and the electrical properties obtained from this animal.

For *Alloteuthis*, MRPs for cell sub-types I, II, III, and IV were not statistically different (P> 0.05, Student's *t* test) (Table 6.3); thus no relationship was found between the four different cell types and membrane resting potential.

Cell input resistances, measured by the balanced bridge method, were in the order of 28.6 ± 8 MΩ (mean ± SD, n=15). No statistical difference (Student's *t* test) was observed in the magnitudes of the cell input resistances for the different cell types. The values of apparent input resistances were highest for type II cells and were in the order of 32.2 ± 7.7 MΩ (mean ± SD) with a range of 20-40 MΩ (Table 6.3). This could be a reflection of the cell size, as cell type II was predominately located in the outer lobe region of the PCL and generally had a larger cell soma compared to the other cell types.

When comparisons were made between spike amplitudes, no statistical differences were found between the cell types (P> 0.05, Student's *t* test). The highest value for spike amplitude was for cell type I, 19.0 ± 11.8 mV (mean ± SD), the SD here is large because the sample size is small (n=9) (Table 6.3).

6.2.3. Spontaneous activity of Alloteuthis subulata PCL cells.

1) Action potentials.

This activity was the most common and occurred in 60 % of the *Alloteuthis subulata* cells sampled (n=95) (Table 6.4). In *Alloteuthis subulata* spontaneous action potentials (also referred to as continuous tonic spontaneous activity) were found to have a mean spike amplitude of 11.4 ± 9.1 mV (mean ± SD n=150) and a large range from 2-70 mV (Table 6.2). The amplitude of the spikes reported here are consistent with other studies where at best, the spike amplitudes were 5 mV (Miyan & Messenger, 1995). The small size of spikes, recorded in some cells, may reflect the nature of the soma activity. The cell soma

of an invertebrate neuron does not carry a regenerative action potential, i.e. the axon spike does not actively invade the soma, instead there is only passive conductance into the soma. The amplitude of the spike will therefore be dependent on how far away the electrode is from the nearest site of spike activation in the cell, which is unknown.

The majority of the cells that exhibited continuous spontaneous action potentials, had firing frequencies of between 1 and 12 Hz (Fig 6.2). These cells were identified as motoneurons, therefore this finding is consistent with the fact that in isolated squid skin the chromatophores show maximum expansion when stimulated at 12-15 Hz (Florey, 1966). On only a few occasions did the spike amplitude overshoot the zero membrane potential point. The shape and amplitude of the action potential varied from a very rapid rising, short duration potential with a large spike amplitude (Fig 6.2a), to a slow rising potential with an after hyperpolarisation and small spike amplitude (Fig. 6.2b). These two forms probably reflect different recording locations within the cell. The former are probably from a location within or near the axon, therefore nearer the spike initiating zone, and the latter from sites within the soma or dendrites, therefore the action potentials seen would be an attenuated form. This interpretation is partly supported by the observation that the axon type spike was generally seen in recordings from the neuropil areas whereas the wider type soma spike was more often seen in recordings from the cell body areas.

By applying pulsed hyperpolarising or constant hyperpolarising current to a PCL cell displaying continuous tonic action potentials, the action potentials could be stopped, or the firing rate slowed, and spike amplitude increased (Fig.6.3a). Applying constant hyperpolarising current to a firing cell sometimes revealed other types of activity; bursting activity in 34% of the cells (n=95) and excitatory postsynaptic potentials (EPSPs) in 30% of the cells (n=95) (Table 6.5). Some of the revealed EPSPs initiated action potentials at their peaks (Fig. 6.3b).

2) Silent cells.

6% of the total *Alloteuthis* PCL cells sampled (n = 95) were silent (Table 6.4). These cells were either in-excitable, showing no activity with injected

current or antidromic stimulation from the pallial nerve, or excitable by the injection of a depolarising pulse which evoked an action potential (Fig 6.4). In some silent PCL cells, the evoked action potentials were single events, even with an increase in the depolarising current (Fig 6.4a), while in other cells, even with a small depolarising current, trains of action potentials were evoked (Fig 6.4b). The amplitude of the evoked spikes ranged from 35 to 5 mV with a mean of 12 mV ± 8 mV (± SD n = 266).

Silent cells have been reported in the PCL of the octopus (Miyan & Messenger, 1995) but the effects of applied current on these cells were not reported, neither were the numbers or percentages of these cells found.

3) Irregular bursting activity.

Spontaneous irregular bursting activity, in the form of action potentials generated at irregular intervals (Fig 6.5), was observed in 16% of the total cells sampled (n=95) in the PCL of *Alloteuthis* (Table 6.4). The amplitude of the spikes generated, the interburst period between spike bursts, and the number of spikes per burst differed amongst different cells. When constant hyperpolarised current was passed into the bursting cell, the number of spikes per burst increased as did the interburst period (Fig. 6.5b). Like the tonic spontaneous cells (1) above), when a constant hyperpolarised current was applied, bursting cells sometimes displayed excitatory postsynaptic potentials (EPSPs) (Table 6.5).

4) Regular bursting activity.

Spontaneous regular bursting activity, during which action potentials occurred at regular intervals (Fig 6.6), was observed in only 5% of the total cells sampled in the PCL of *Alloteuthis* (n = 95) and was the rarest activity seen (Table 6.4). Regular bursting was also revealed in 3% of the cells that displayed tonic action potentials, when they were injected with hyperpolarising current (Table 6.5).

Each PCL cell so far recorded with this activity (12 altogether) appears to have its own distinctive period and rhythm (Fig 6.6). One cell had a regular

number of 5 spikes in adjacent bursts (Fig 6.6a). Figure 6.6b reveals an example of a cell with oscillating membrane properties, in which the cell potential varied almost sinusoidaly with slow depolarisation and hyperpolarisation phases. The bursts are fairly regular, in that spike numbers are between 3 and 2 in adjacent bursts.

Regular bursting has never been seen before in the cephalopod PCL. However, oscillating neurons, firing in regular bursting patterns, located possibly at the dorsal side of the pallio-visceral lobe in the octopus whole brain, have been reported (Laverack, 1980). There is no indication as to what behavioural activities these cells might govern, although respiratory functions are reported to be generated in this area (Boycott, 1961). The significance of this activity is unclear but these cells could be involved in the pulsating activity of chromatophores on the skin.

5) Excitatory postsynaptic potentials (EPSPs).

Large depolarising potentials that caused fast shifts in the membrane potential, were termed EPSPs (Fig. 6.7a). 13% of the total cells sampled (n= 95) in *Alloteuthis* displayed this activity (Table 6.4). When a cell displaying EPSPs was hyperpolarised by applying constant current, action potentials on the peaks of the EPSPs were no longer apparent (Fig. 6.7b). In some tonically active and bursting cells that were hyperpolarised, EPSPs were sometimes observed (Table 6.5). EPSPs appeared randomly and had different durations. Some had relatively steep rise times, while others had gradual rise times (Fig. 6.8). EPSP amplitude ranged from 2 to 40mV (Fig. 6.8). These large events could be the result of an increase or summation of excitatory synaptic potentials and/or a decrease in inhibitory synaptic potentials resulting in a very large EPSPs. IPSPs were less commonly observed. It was noted that more EPSPs were present among cells in slices that had not been left to recover for a long period of time.

Because spontaneous EPSP activity was very noticeable in many PCL neurons, and represents synaptic connectivity between cells, this type of activity was studied further.

5a) Spontaneous EPSPs

Spontaneous EPSPs were composed of events that occurred alone or in clusters, where they often summated (Fig. 6.7a). Both single and summated spontaneous EPSPs could trigger action potentials (Fig. 6.7a). Spontaneous EPSPs from a given cell could vary during a recording session, both in amplitude and in frequency of the events. In the majority of cells, with increasing duration of impalement, there was a decrease in the number of spontaneous EPSPs and an increase in the amplitude of the EPSPs seen (Fig. 6.9a & b); this effect occurred without any change in the MRP of the cell. The decrease in the number of EPSPs indicates that the impulses from the presynaptic cell decreased, perhaps due to the deteriorating condition of the slice with time. The increase in the amplitude of the EPSPs could be due to the activation of another synaptic input, positioned closer to the recording electrode. The EPSPs generated by this synapse would be less attenuated by the time they reached the recording electrode.

5b) Voltage dependence of spontaneous EPSPs.

When the cell membrane potential was experimentally manipulated to holding potentials of -55, -65, and -90 mV by injection of a negative current, the incidence of large EPSPs was found to increase with increasing holding current (Fig. 6.10) such that the mean EPSP amplitude was strongly correlated with membrane potential (regression coefficient =0.96).

The mean frequency of EPSP events, as shown in Fig. 6.10, was 0.67 ± 0.3 EPSPs per second (mean EPSPs/sec ± SD) at a holding potential of -55mV, 0.67 ± 0.47 EPSPs per second (mean EPSPs/sec ± SD) at a holding potential of -65mV, and 0.88 ± 0.6 EPSPs per second (mean EPSPs/sec ± SD) at -90mV holding current. These means were not significantly different even though the amplitude of the EPSPs increased with hyperpolarisation. This is what one would expect as the number of EPSPs is governed presynaptically and would not be effected by any postsynaptic alteration of the holding potential of the cell.

5c) Evoked EPSPs.

EPSPs could be evoked in PCL cells by electrical stimulation of the lateral basal lobe tract. These evoked responses, recorded intracellularly from PCL cells, varied in their rise time and amplitude (Fig. 6.11). The shape and size of the EPSPs varied depending on the location of the synapse in relation to the recording electrode. When a repeated stimulus was applied to the LBL tract, EPSPs occurred at a constant latency from the stimulus, while in other cells EPSPs occurred at different latencies from the stimulus artefact (Fig.6.12a). This may be due to the involvement of more than one synapse with these cells. In one particular cell the latency of EPSPs occurred most frequently at 7 msec from the stimulus artefact (Fig. 6.12b).

5d) Voltage dependence of evoked responses.

When the cell membrane potential was altered, from between -50 mV and -90 mV, the amplitude of evoked EPSPs increased but the EPSP shape stayed constant (Fig. 6.13). As with the spontaneous EPSPs, this increase in EPSP amplitude was proportional to the change in the membrane potential, with a regression coefficient of 0.99.

5e) Changes in evoked EPSPs with stimulus strength.

For low amplitude stimuli, the cell did not always show a response. However, if the strength of the stimulus was increased, this sometimes evoked an EPSP. The increase in the EPSP size with increasing stimulus of the lateral basal tract was probably due to the recruitment of additional fibres with increasing stimulus strength.

6.2.4. Spontaneous activity of the different morphological cell types in the PCL of Alloteuthis subulata.

1) Action potentials

Spontaneous action potentials were observed in more cells than any other type of activity and more predominantly in cell type I (Table 6.4):

70% in cell type I (n = 10)

60% in cell type II (n = 50)

55% in cell type III (n = 33)

100% in cell type IV (n = 2)

From a total *Alloteuthis* PCL cell sample size of 95 cells (Table 6.4). The low number of cell type IV being dye stained does not give an accurate estimation of the activity of this cell type.

It was found that 28% out of the 70 % of cell type I cells that displayed repeated action potentials, when hyperpolarised, displayed EPSPs, and 24% out of 62% of cell type II and 16% out of 55% in cell type III did the same (Table 6.5). When hyperpolarised, bursting activity was displayed in 38% of tonically active type III neurons (Table 6.5).

2) Silent activity

This was not a common occurrence and was displayed most predominately in cell type I (Table 6.4):

10% of cell type I (n = 10)

6% of cell type II (n = 50)

6% of cell type III (n = 33)

none in cell type IV (n = 2)

(Table 6.4)

3) Irregular bursting activity

This activity was predominately seen in cell type II (Table 6.4).

10% of cell type I (n = 10)

20% of cell type II (n = 50)

12% of cell type III (n = 33)

none in cell type IV (n = 2)

(Table 6.4)

When these cells types displaying bursting activity were hyperpolarised, 25% of type III cells and 27% of type II cells displayed EPSPs (Table 6.5).

4) Regular bursting

Regular bursting activity was observed only in cell sub-type II and constituted 10% of the cells total activity (Table 6.4). 10 cells that also displayed regular bursting activity were not successfully dyed, thus it would be presumptuous to say that only cell type II is capable of showing this behaviour. 3% of the tonically firing neurons of cell type II, when injected with hyperpolarised current, showed regular bursting activity (Table 6.5).

Cell type II is the commonest cell type found (Table 6.1), has extensive arborizations and is predominantly situated in the outer region of the lobe (Chapter 4). The possibility that this cell type governs some rhythmic chromatophore activity on the skin needs further investigation and clarification.

5) Excitatory postsynaptic potentials (EPSPs)

This activity was predominately seen in cell type III (Table 6.4):

10% of cell type I (n = 10)
4% of cell type II (n = 50)
27% of cell type III (n = 33)
none in cell type IV (n = 2)
(Table 6.4)

As already mentioned, EPSPs occurred predominantly in cell types I and III (Table 6.4), but no obvious differences were observed in spontaneous EPSPs in neurons from different areas within the PCL.

EPSP activity was displayed in 28% of the tonically active cell type I cells when they were hyperpolarised, 24% of cell type II, 16% of cell type III, and 50% in cell type IV (Table 6.5).

6.2.5. Coupling between PCL cells.

Electrical coupling occurs in many different sorts of tissue and permits intracellular communication between two or more cells. For neurons, such coupling is useful in providing a very rapid contact between cells or for co-ordinating the activity of a population of cells. Electrical coupling between PCL

neurons was not extensively investigated in this study and was examined in a few cases only. Recording electrodes were placed into two separate cells. An example is shown in Fig. 6.14 where a chromatophore motoneuron (cell 1) was impaled and showed spontaneous tonic activity. The second recording electrode, filled with LY, was placed carefully in the vicinity of cell 1 and a second cell was impaled (cell 2). Immediately following this, the second cell fired action potentials which were very closely associated with those seen in cell 1. Cell 2 spike amplitude was three times smaller than in cell 1 (Fig. 6.14a). These cells appeared to be strongly coupled. Small, irregular depolarising potentials could be seen in cell 1 and 2 that were not visible in cell 1 before impalement of cell 2. These resembled localised potentials (EPSPs) (Fig. 6.14a). Some of these potentials did not occur in cell 2 but were apparent in cell 1 (Fig. 6.14a). An action potential could be antidromically evoked in both cells by stimulating the pallial nerve (Fig. 6.14b), but it was not certain that cell 2 was also a chromatophore motoneuron as this could be due to it being coupled with cell 1. Unfortunately cell 1 was lost before current could be injected into the cell in order to observe any corresponding effects in cell 2. Some dye was injected into cell 2 and dye coupling was observed in these two cells, another possible indicator of electrical coupling. This experiment indicates that some electrical coupling is present in the PCL but its overall occurrence and influence is not yet known.

6.3 Summary

1) This section has set out to provide information on the electrical activities of PCL cells and to relate this information to the morphology of each cell. Identification of any differences between the physiology of the four cell types describe in Chapter Four, could therefore be made.

2) Intracellular recordings were obtained from PCL cells and these were subsequently filled with Lucifer Yellow.

3) Mean membrane resting potentials (MRPs) of PCL cells in *Alloteuthis* were found to be −37.5 mV. There was a significant difference in the MRP of cells between *Alloteuthis*, *Loligo*, *Eledone*, and *Octopus*. The MRP of the four cell types did not differ significantly.

4) Intracellular recordings revealed 5 different types of spontaneous activity in the PCL cells of *Alloteuthis*;

   A; Repeated action potentials.

   B; Silent cells.

   C; Irregular bursting.

   D; Regular bursting.

   E; Excitatory postsynaptic potentials (EPSPs).

5) The most predominant activity seen in the PCL cells was repeated spontaneous action potentials, seen in 60% of the cells sampled. Irregular bursting was the second commonest activity seen (16%) then, EPSPs (13%), silent activity (6%) and regular bursting activity (5%).

6) Cell type I and III displayed every type of activity except regular bursting activity, which was only displayed in cell type II. Cell type IV, however, displayed only repeated action potential activity.

7) EPSPs were predominantly displayed in cell type III, 27% of this cell type showed this activity, which is more than double that seen in the other cell types.

8) When PCL cells were hyperpolarised a number of effects were seen;

   A; Tonically active cells stopped firing.

   B; EPSP and bursting activity was seen in tonically active cells.

C; Bursting cells displayed EPSPs.

D; EPSPs increased in amplitude.

9) An electrical connection between the lateral basal lobe (LBL) and the PCL was established for the first time, thereby confirming previous morphological data. The LBL tract was stimulated, and this resulted in evoked EPSPs in the PCL cells. The EPSPs varied in shape and amplitude, from cell to cell, depending on the location of the recording electrode within the PCL.

10) Electrical coupling was shown for the first time in PCL cells. This result is consistent with the dye coupling results shown in this thesis and with previous work.

11) There are some differences in the activity shown among the different cell types described previously. Cell type II for example, was the only cell to display regular bursting activity, and cell type IV displayed only action potentials. However, a larger sample size and further investigation is needed to show how the different cell morphologies and electrical activities might effect the skin colour of the animal.

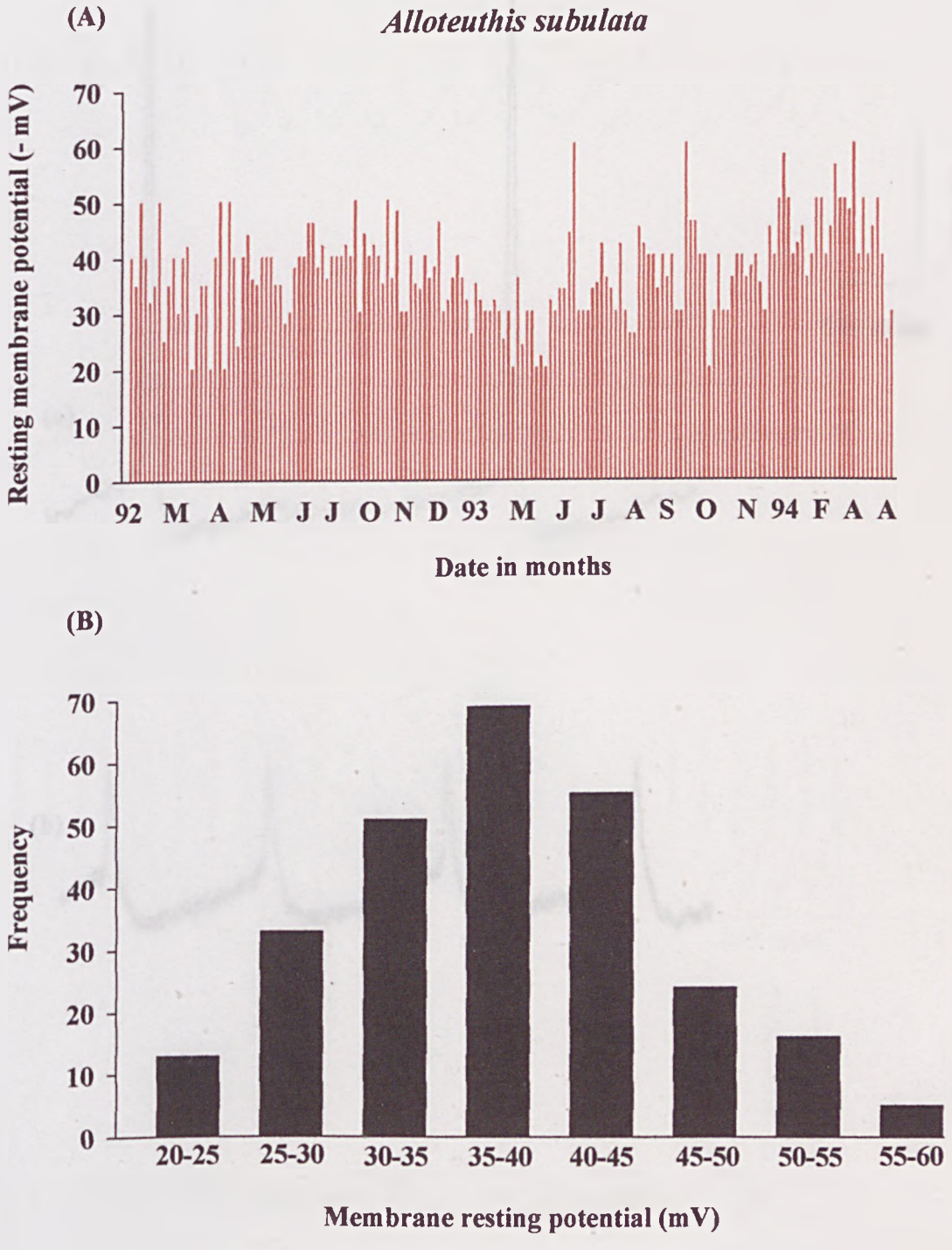


Fig. 6.1 Membrane resting potentials of *Alloteuthis* PCL neurons. (a) A graph showing the seasonal variation of the MRPs. (b) A frequency distribution graph, showing normal distribution, reveals that MRPs ranged from -20 to -60mV with a mean value of -37.5mV.

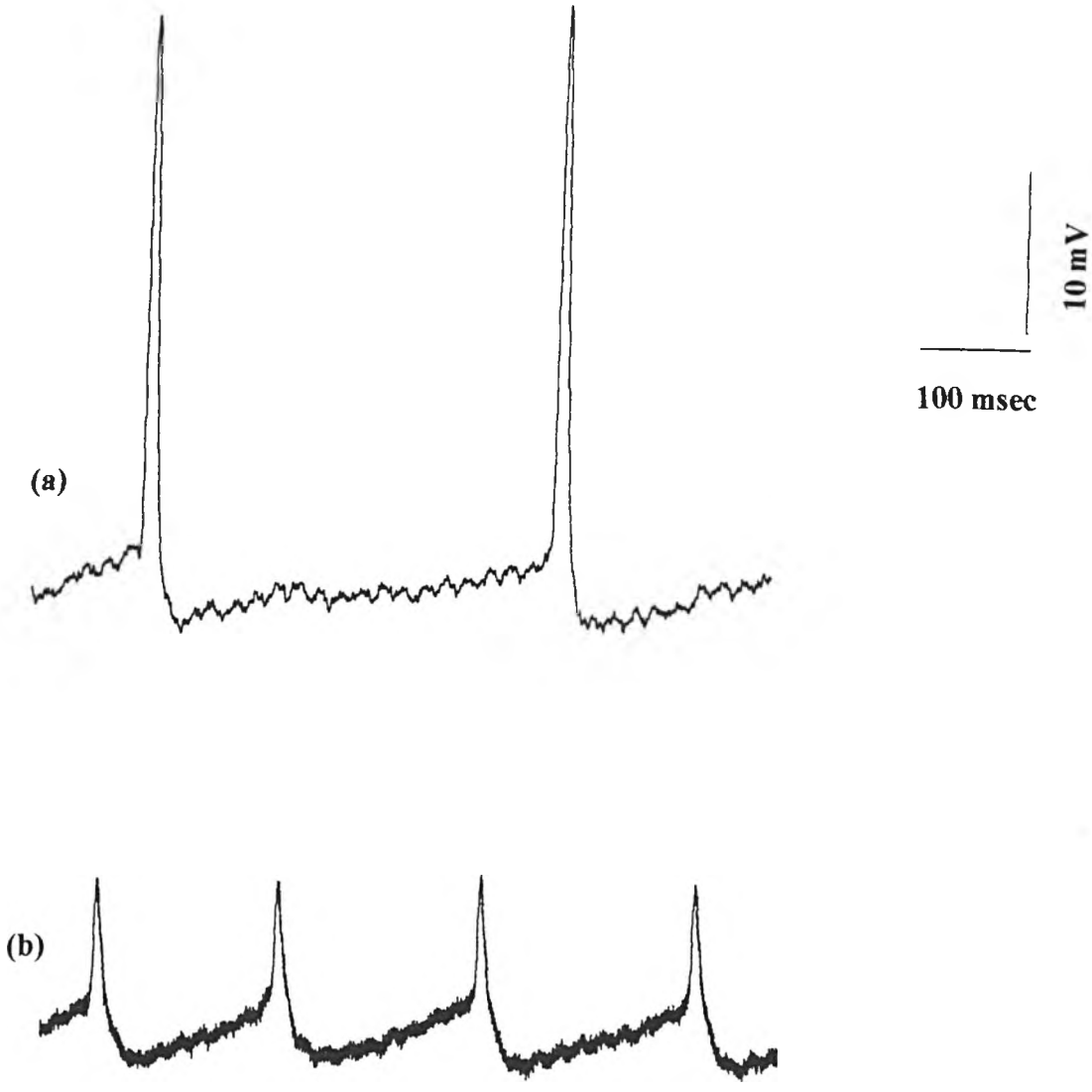


Fig 6.2 Examples of action potentials taken from two separate PCL motoneurons from *Alloteuthis*. (a) An example of a rapidly rising action potential with a large amplitude; this cell was located near the neuropil area and the recording was probably made from the cell axon. (b) An example of a slow rising small amplitude action potential with a large hyperpolarisation, this cell was located near the periphery.

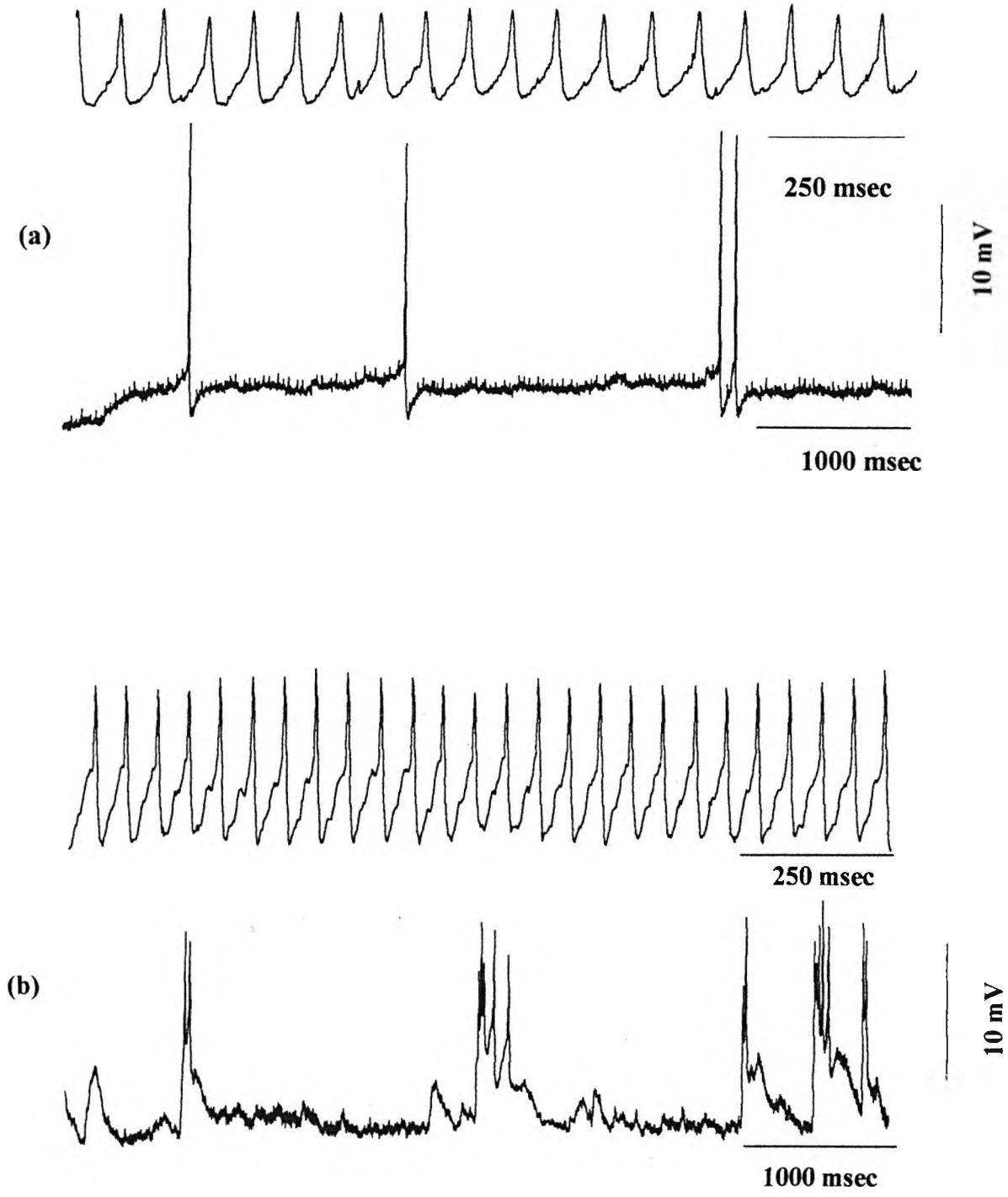


Fig 6.3 The effects of injecting a hyperpolarising current on spontaneous tonic PCL cells. (a) A tonic cell was hyperpolarised from a membrane potential of -36mV to -50 mV. This caused an increase in the spike amplitude and a decrease in firing frequency. (b) A tonically firing cell was hyperpolarised from -30mV to -

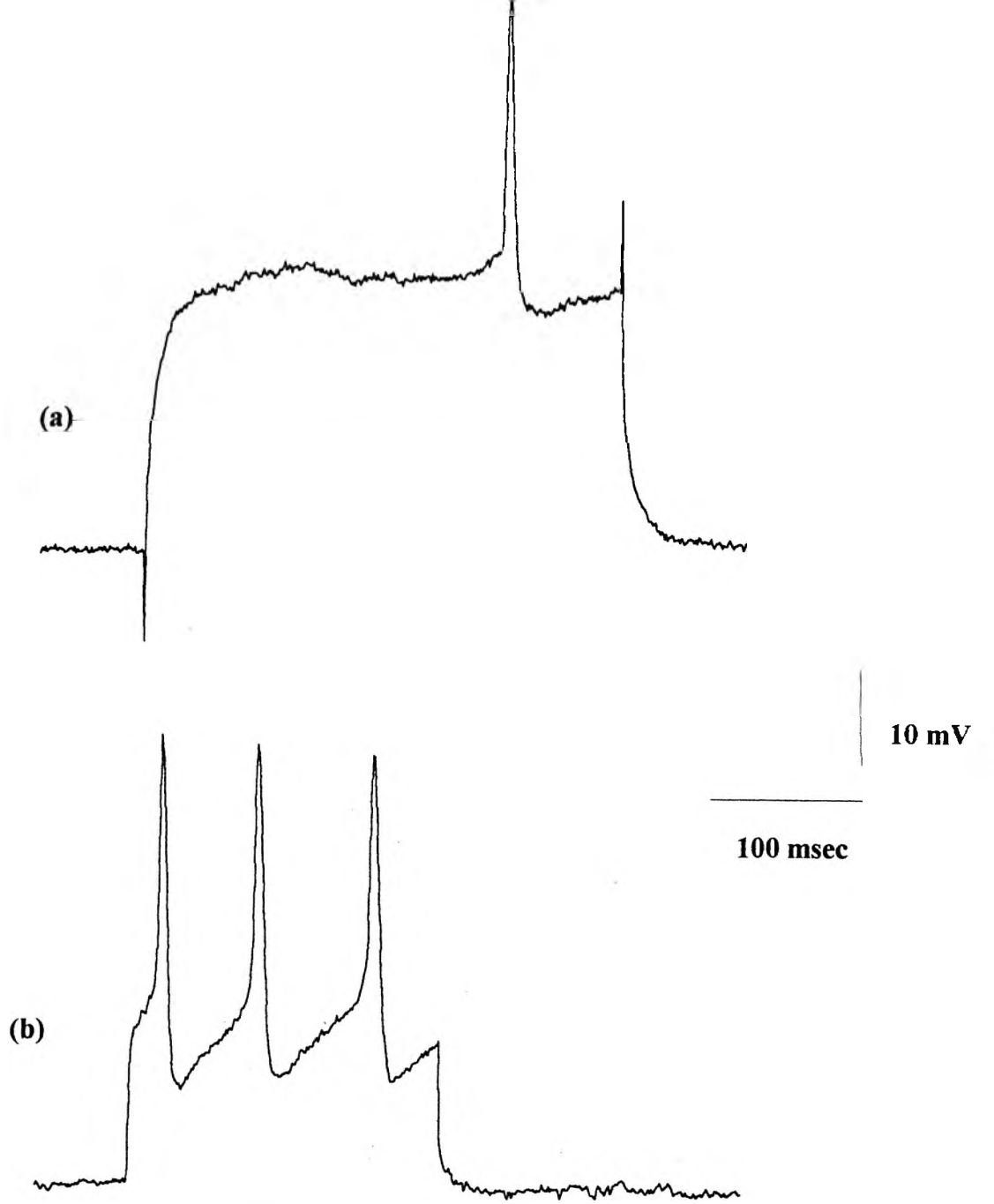


Fig. 6.4 (a) A single action potential evoked in a PCL neuron on injection of a pulsed depolarising current. The cell was of type II morphology (see Chapter Four), was located in the inner cell layer and had a MRP -46 mV. (b) A train of action potentials were evoked in an otherwise silent cell type I located in the outer cell layer on injection of depolarising current.

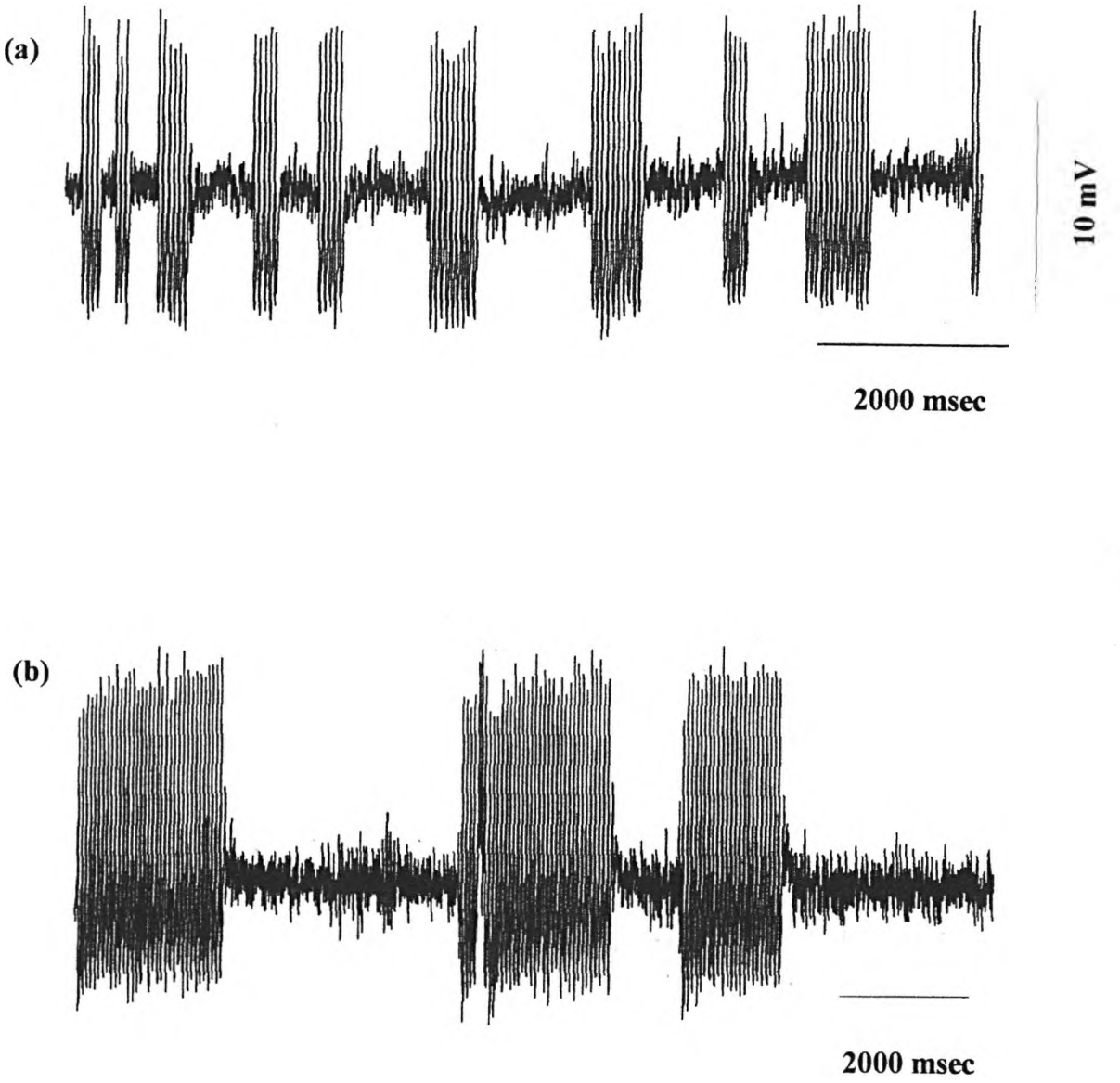


Fig 6.5 The effect of hyperpolarising a PCL cell displaying irregular bursting activity. (a) A bursting PCL type II cell showing spontaneous activity at a MRP of -36mV. (b) When the cell was hyperpolarised to -45mV the number of action potentials per burst increased as did the time between the bursts.

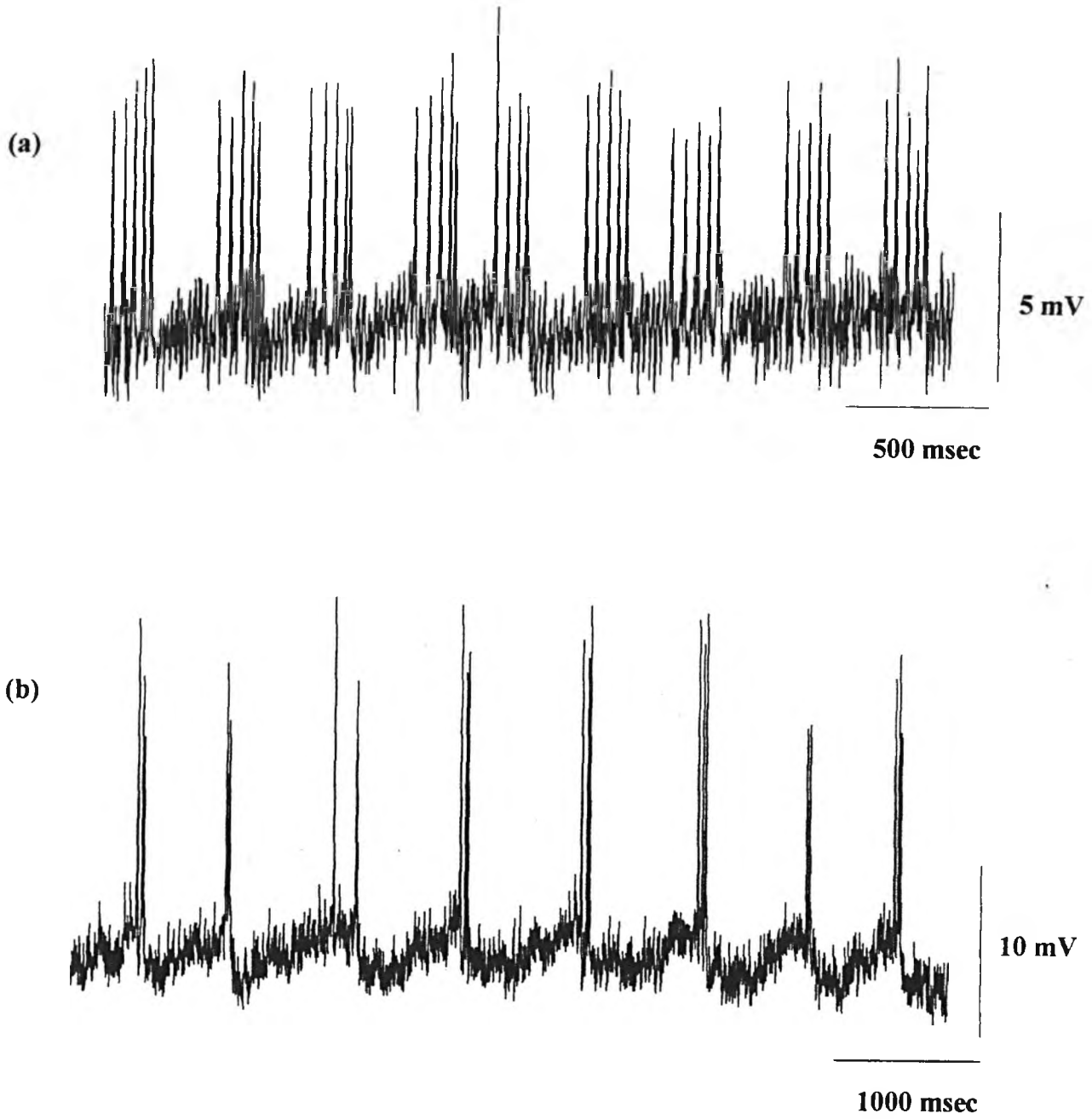


Fig 6.6 Examples of two PCL cells displaying regular bursting activity. (a) This cell was located in the periphery of the PCL and was of the morphological type II cell (Chapter Four). Note the consistency of the five spikes per burst of activity. (b) This cell has regular bursting behaviour with an underlying membrane oscillation. Both of these cells were antidromically identified as motoneurons.

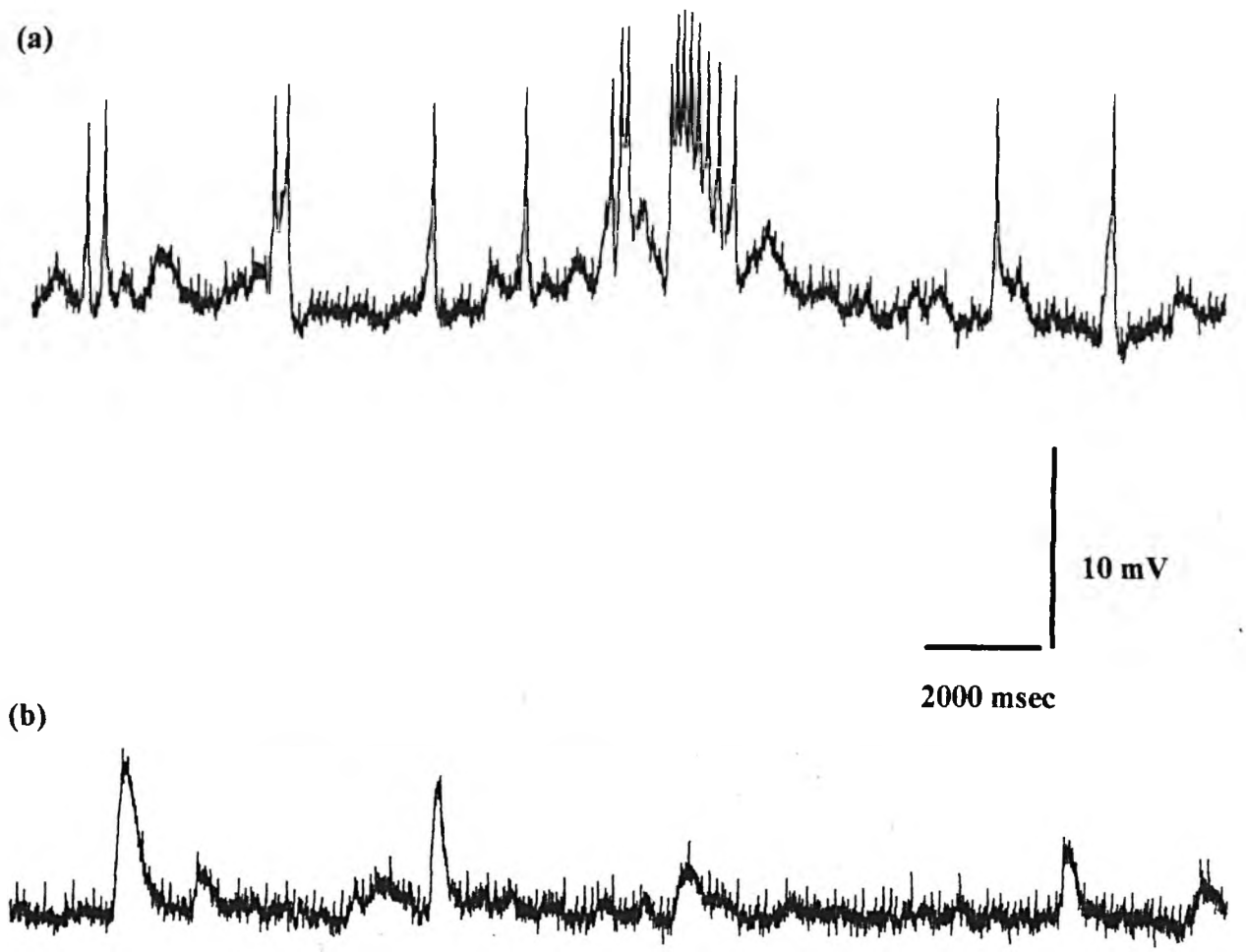


Fig 6.7 The effect of hyperpolarising a PCL neuron displaying EPSP activity. (a) In this cell, positive shifts in the membrane potential occurred which sometimes were associated with a burst of action potentials. (b) When the same cell was hyperpolarised from a membrane potential of -36mV to -50mV, the EPSPs became infrequent and the bursts of action potentials on the peak of the EPSPs were no longer present. This cell was located in the mid cell layer and was of cell type III.

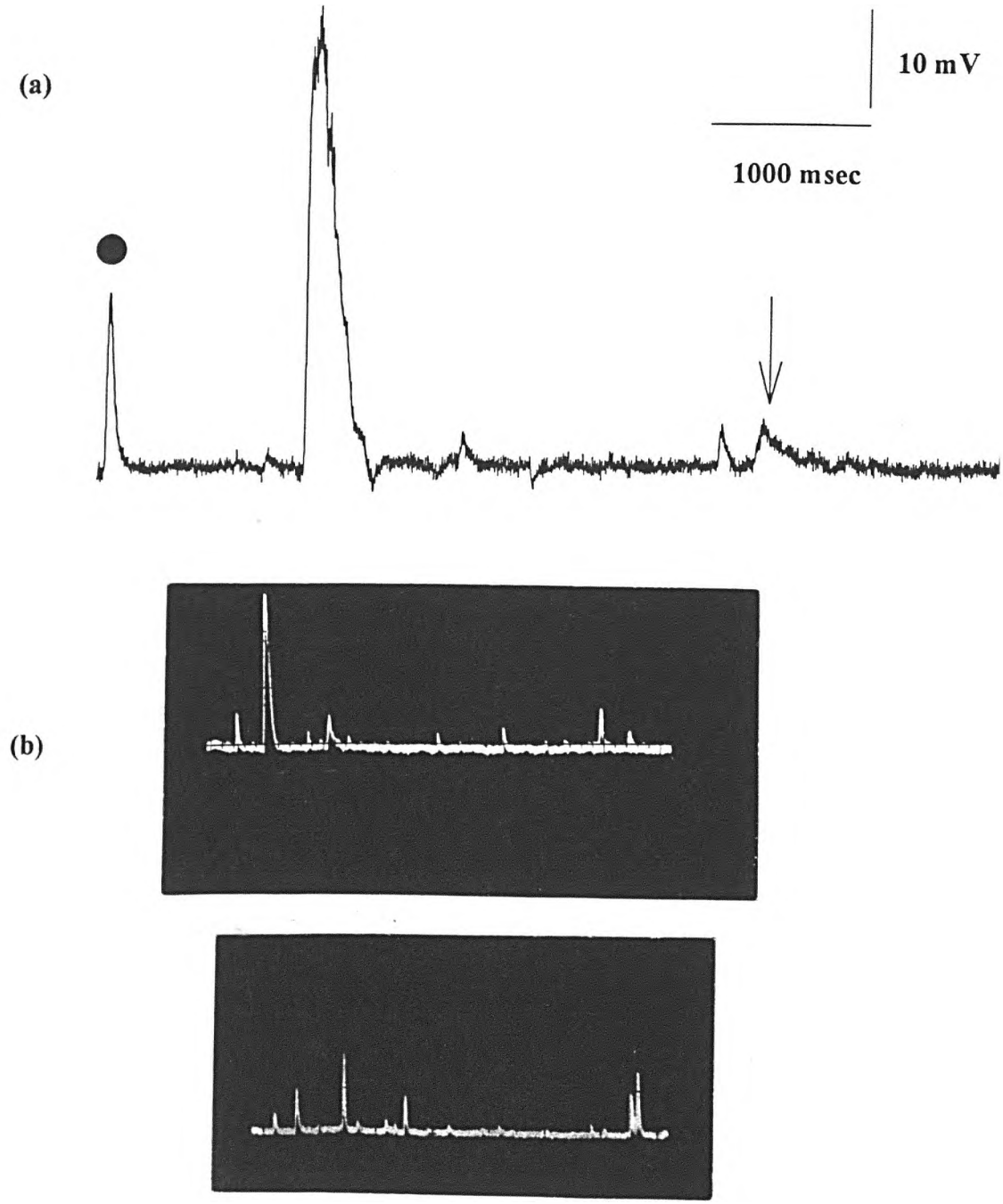


Fig 6.8 (a) A cell in the PCL located in the mid cell layer showing EPSP activity. The EPSPs occurred randomly and had different rise times (*V/T*). One EPSP had a steep rise time of 0.11 mV/sec ● while another had a relatively slow rise time of 0.02 mV/sec ⟶ (b) Photograph of the oscilloscope recording from the same cell. Note the difference in the amplitudes and shape of the EPSPs during a 7 second recording period.

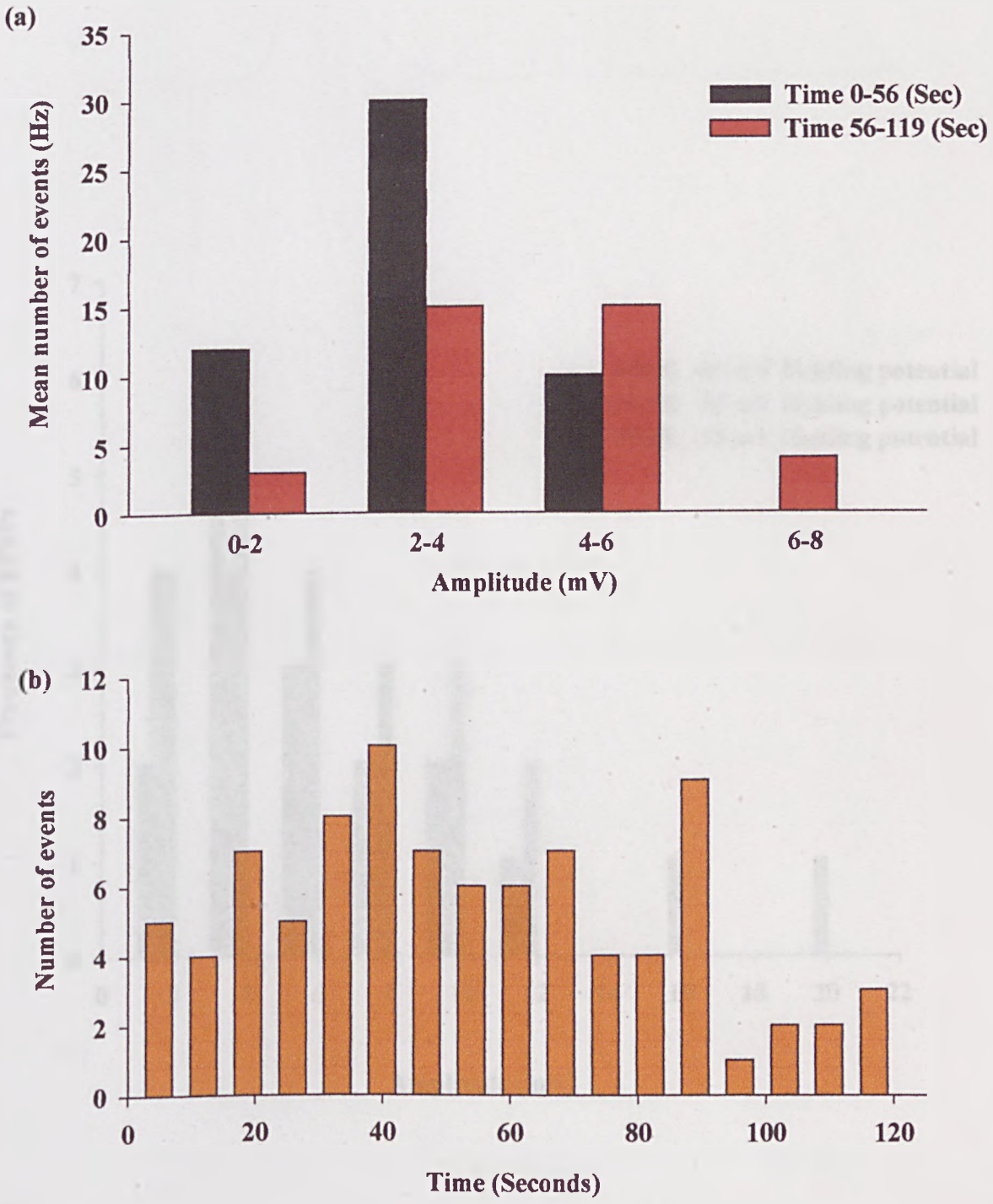


Fig 6.9 (a) A graph showing the changes in the amplitude of EPSP events in a PCL cell during impalement, with respect to the effect of time on EPSP amplitude. The amplitude of the EPSPs increased while the MRP remained constant at -40 mV throughout the experiment. (b) Graph showing that in the same cell, the number of EPSP events decreased with time.

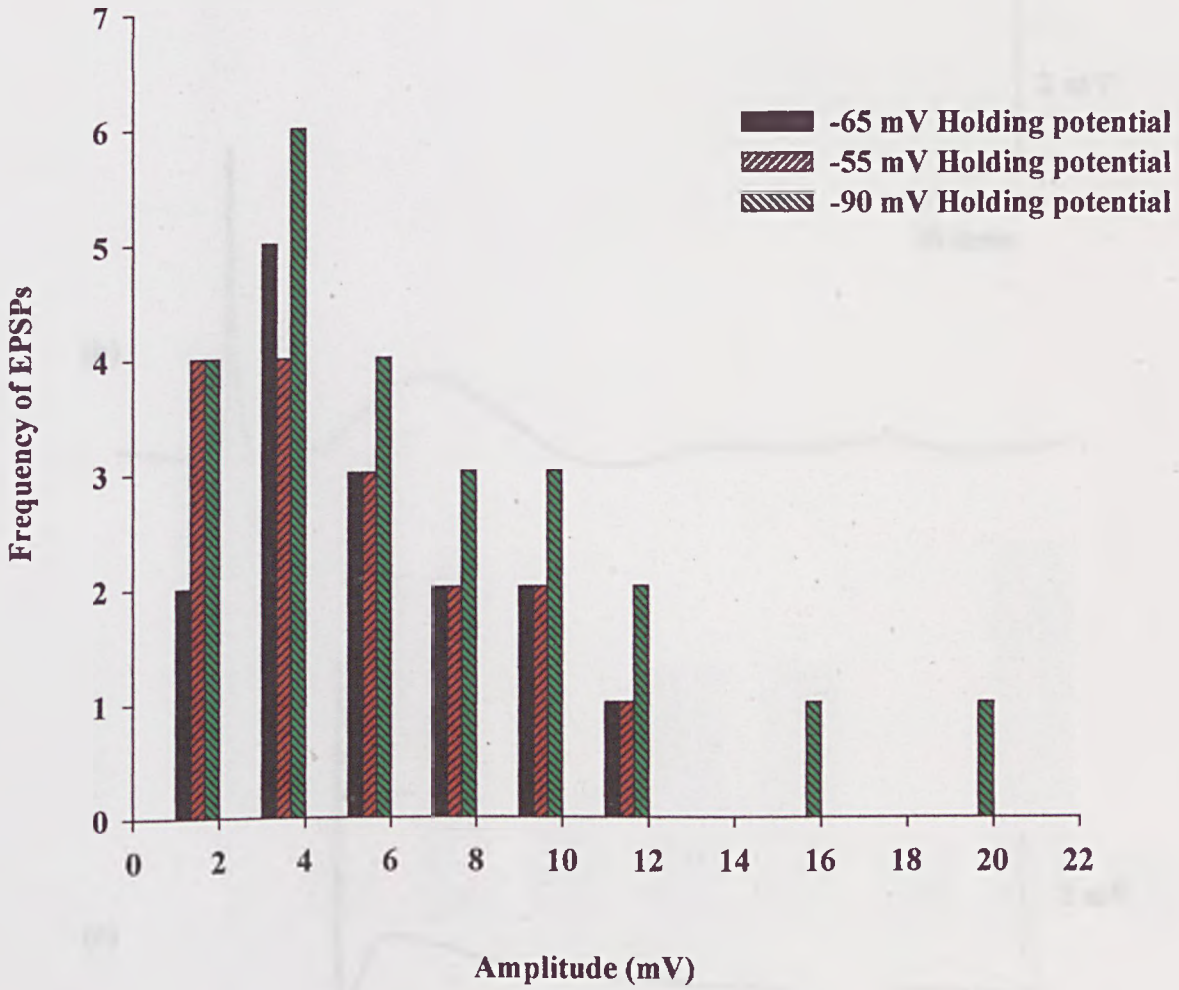


Fig 6.10 Frequency distribution graph showing the voltage dependence of spontaneous EPSPs. The frequency of larger amplitude EPSPs increased with increasing hyperpolarisation of the membrane potential from -55 mV to -90 mV.

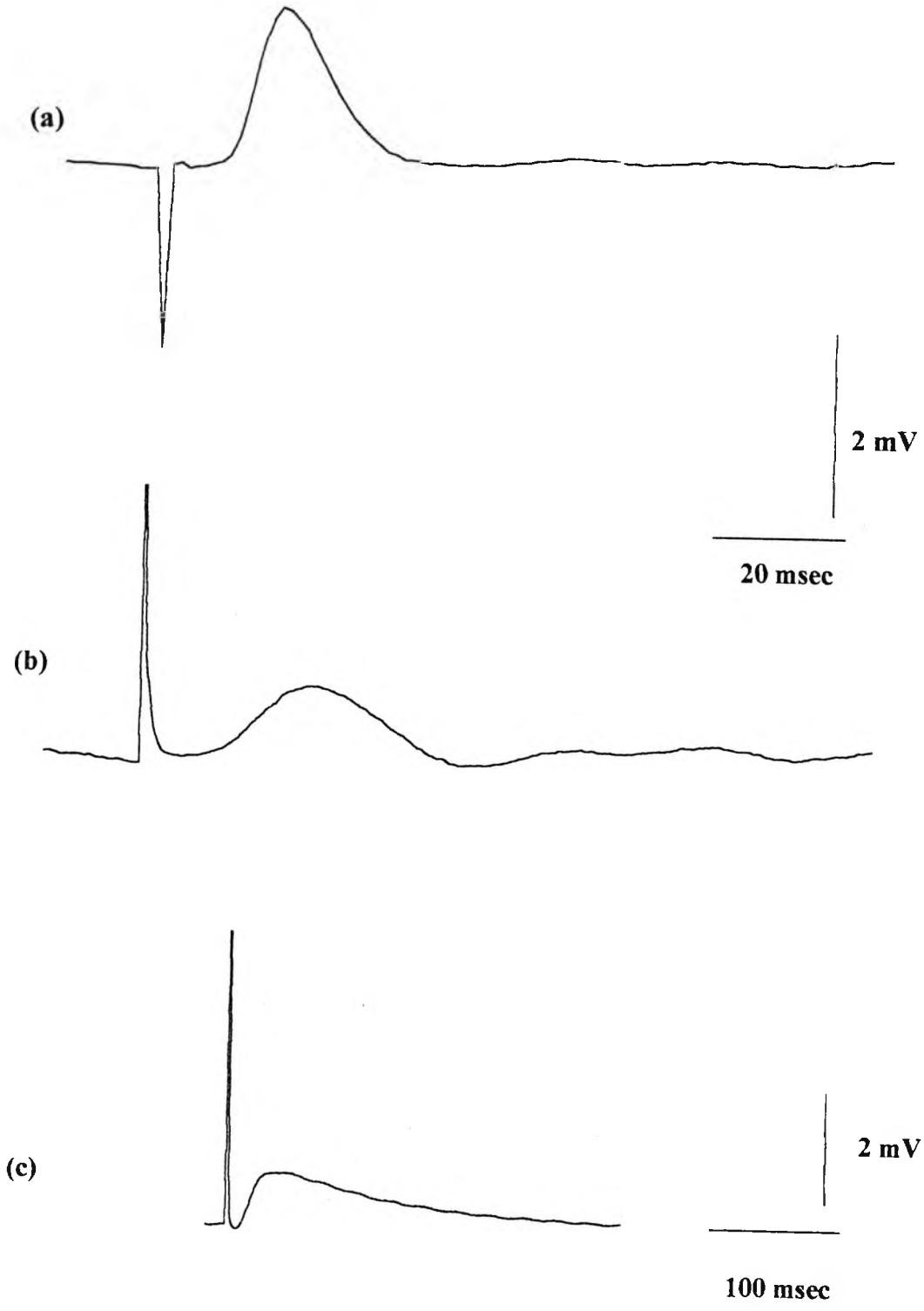


Fig 6.11 Examples of EPSPs evoked by stimulating the LBL tract. The EPSPs vary in amplitude and shape. Cells (a) & (b) were located in the mid cell layer of the PCL and had MRPs of -35 to -45mV. Cell (c) was located in the inner cell layer of the PCL, near the neuropil.

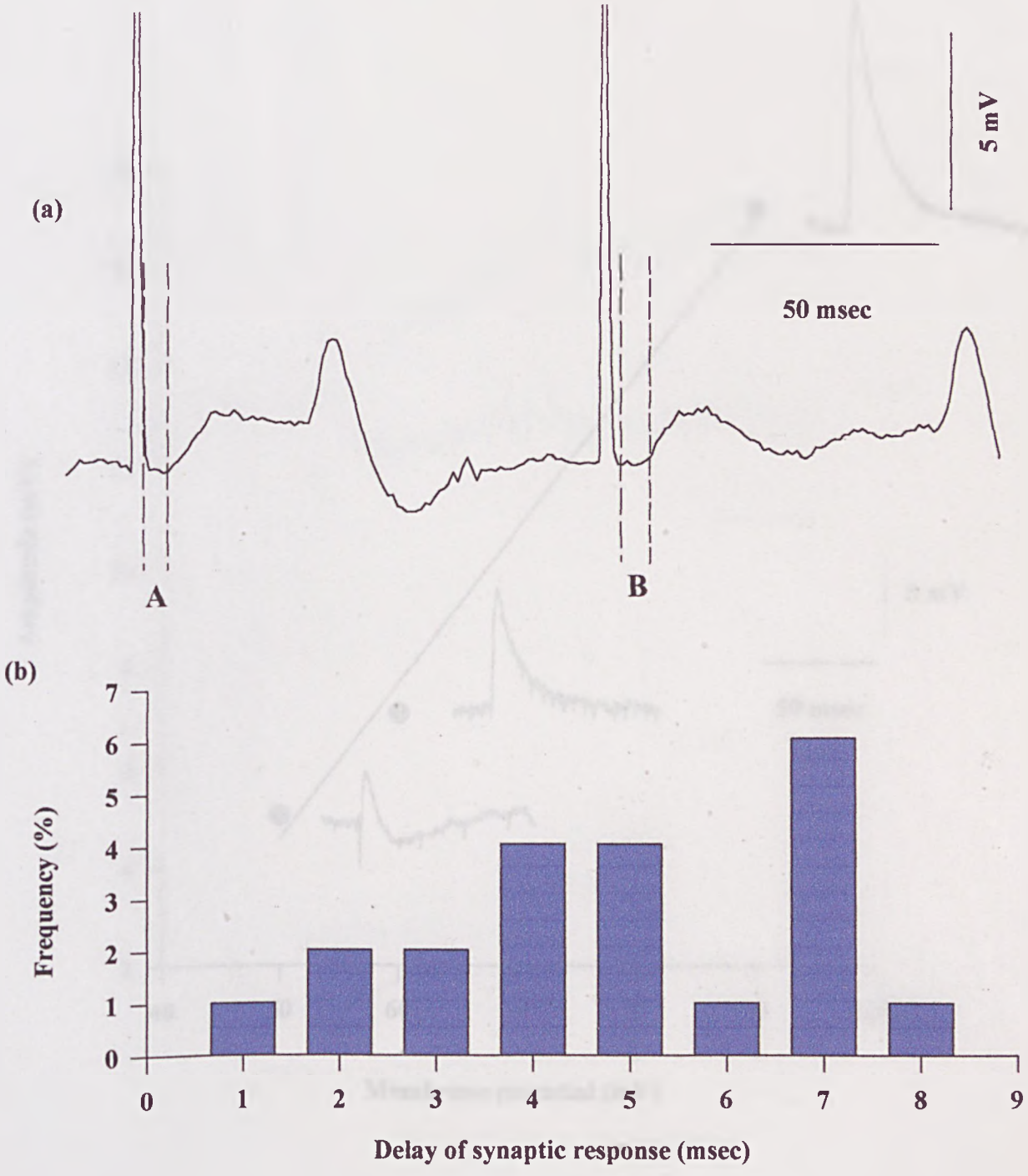


Fig 6.12 (a) A tonically active PCL cell displaying evoked EPSPs on repeated stimulation of the LBL tract. The latency of the response varied (A) & (B) indicating the involvement of more then one synapse with this cell. (b) When the frequency of synaptic delays of the response were plotted for this cell, the most common delay of the EPSPs occurred at 7 msec.

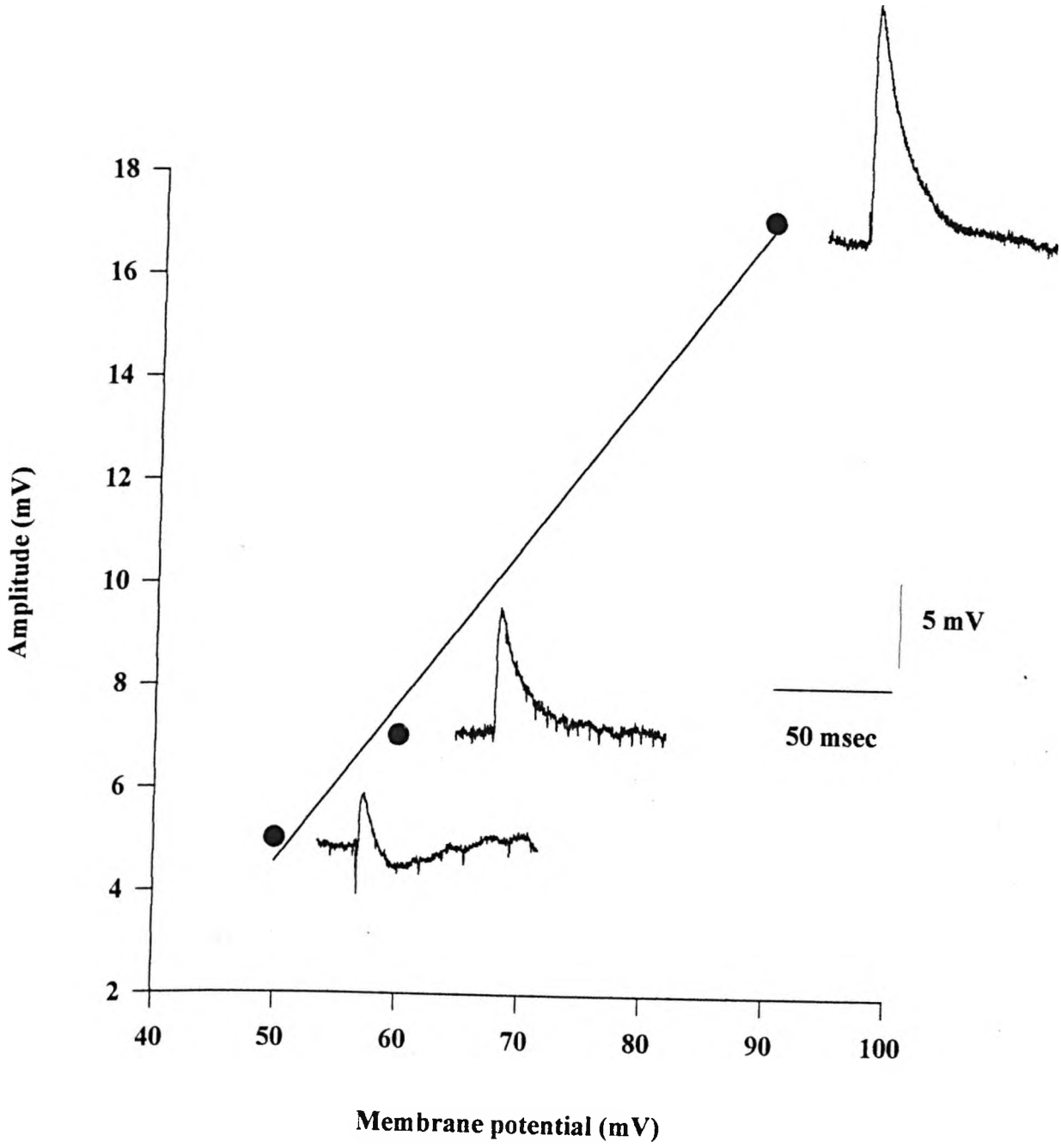


Fig 6.13 Graph with measured intracellular recordings, showing the voltage dependence of EPSPs in PCL neurons, evoked by stimulation of the LBL tract. Evoked EPSPs show a strong voltage dependence with an increase in the amplitude of the EPSPs on increase of the membrane holding current (Correlation coefficient $r^2 = 0.99$). This cell was located within the inner cell layers of the PCL and had a membrane resting potential of -40mV.

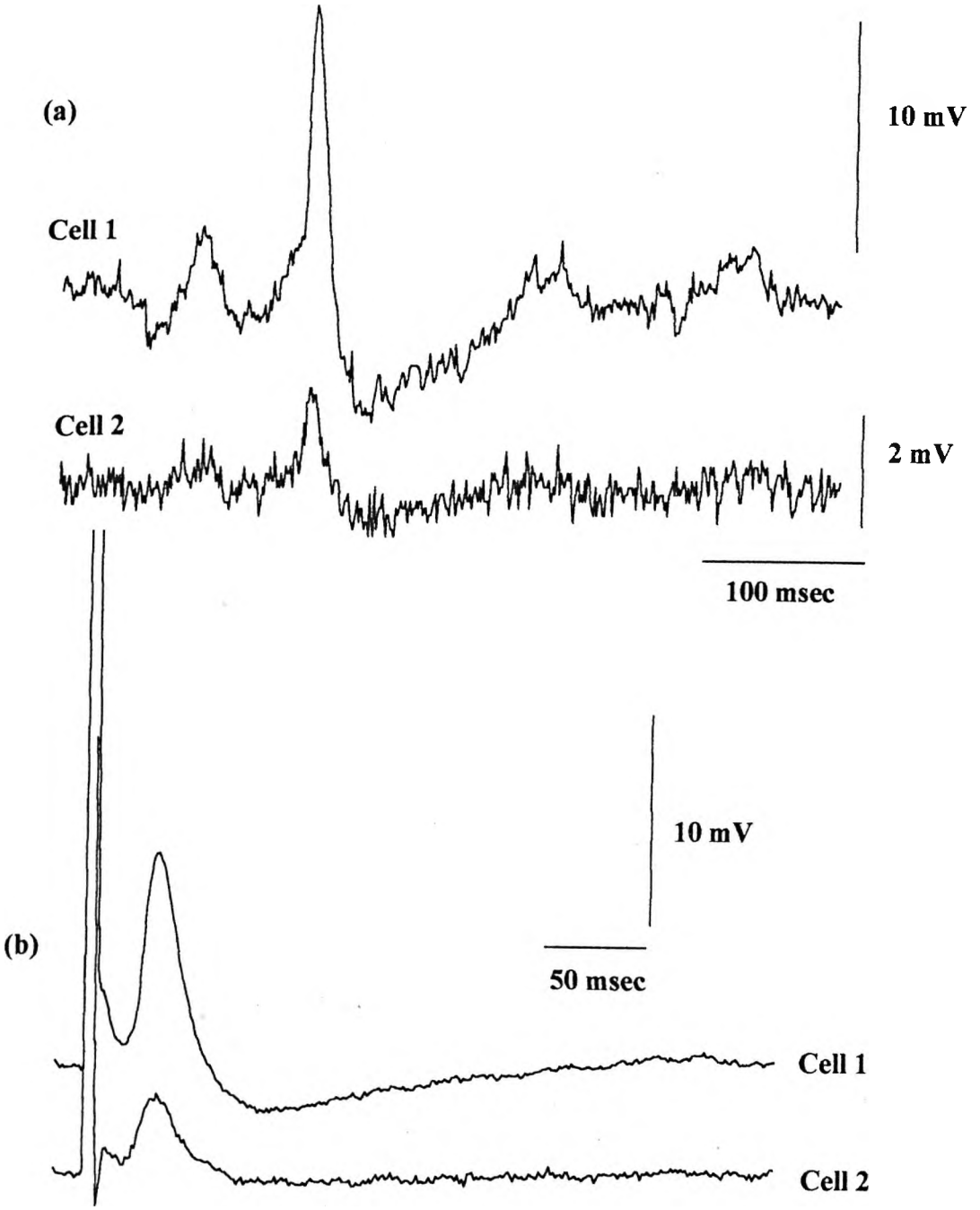


Fig 6.14 (a) Intracellular recordings of two coupled neurons located in the mid cell layer of the PCL. Cell 1 was impaled first and displayed action potentials and EPSP activity which did not always occur in Cell 2. These two neurons were synchronised in their activities throughout the duration of the recording. (b) Antidromic responses in both cells evoked by stimulating the pallial nerve.

Table 6.1. The total number of intracellular recordings from the PCL of four species of cephalopod during the period from 19th March 1992 to 12th August 1994

Animal	No. intracellular recordings	No. PCL cell types identified by intracellular dye injection				Total
		I	II	III	IV	
Alloteuthis subulata	266	10	50	33	2	95
Loligo vulgaris	162	2	2	8	2	14
Eledone cirrhosa	42	2	-	3	-	5
Octopus vulgaris	33	-	-	1	-	1
Total	503	14	52	45	4	115

Table 6.2. Differences in the membrane resting potential and spike amplitude of PCL cells among different cephalopod species.

Animal	Resting membrane potential (mV)	Spike amplitude (mV)
Alloteuthis subulata	−37.5 ± 8.4 (266) [−20 to −60]	11.4 ± 9.1 (150) [2-70]
Loligo vulgaris	−40.9 ± 11.0 (162) [−23 to −62]	15.7 ± 13.4 (82) [2-65]
Eledone cirrhosa	−23.8 ± 13.5 (42) [−20 to −56]	13.7 ± 11.4 (39) [2-40]
Octopus vulgaris	−32.1 ± 11.4 (33) [−22 to −59]	10.6 ± 6.21 (17) [2-20]

Values are in means ± SD with numbers of neurons in parentheses and range in brackets.

Table 6.3. Electrical properties of the different cell types in the PCL of *Alloteuthis subulata*

Cell type	Resting membrane potential (mV)	Apparent Input Resistance (MΩ)	Spike Amplitude (mV)
I	−41.8 ± 8.8 (10) [−30 to −56]	24.0 ± 5.2 (3) [18-28]	19.0 ± 11.8 (9) [5-40]
II	−35.7 ± 9.6 (50) [−20 to −60]	32.2 ± 7.7 (5) [20-40]	10.5 ± 7.4 (47) [2-35]
III	−36.1 ± 8.0 (33) [−20 to −60]	29.7 ± 11.6 (7) [15-45]	13.6 ± 7.7 (31) [5-30]
IV	−30.0 ± 0 (2) [−30]	–	11.0 ± 1.4 (2) [10-12]

Values are in means ± SD with numbers of neurons in parentheses and range in brackets.

Table 6.4. Percentage of different neuron types showing electrical activity in the PCL of *Alloteuthis subulata.*

Electrical activity of cells	Total No of cells	%	Cell type							
			I	%	II	%	III	%	IV	%
Silent	6 (95)	6	1 (10)	10	3 (50)	6	2 (33)	6	-	-
Bursting irregular	15 (95)	16	1 (10)	10	10 (50)	20	4 (33)	12	-	-
Bursting regular	5 (95)	5	-	-	5 (50)	10	-	-	-	-
Tonic	57 (95)	60	7 (10)	70	30 (50)	60	18 (33)	55	2 (2)	100
EPSP	12 (95)	13	1 (10)	10	2 (50)	4	9 (33)	27	-	

Values are for number of cells with total number of cells in parentheses

Table 6.5 Percentage of tonically active and bursting cells that displayed a different activity when constant hyperpolarising current was applied in four different cell types of the PCL of *Alloteuthis subulata.*

Cell type	Activity of tonically active cells				Activity of Bursting cells			
	Silent %	Bursting irregular %	Bursting regular %	EPSP %	Silent %	Bursting irregular %	Bursting regular %	EPSP %
I	2 (7)	-	-	28 (7)	-	-	-	-
II	3 (33)	15 (33)	3 (33)	24 (33)	-	-	-	27 (11)
III	5 (18)	38 (18)	-	16 (18)	-	-	-	25 (4)
IV	-	50 (2)	-	50 (2)	-	-	-	-
Total	10 (58)	103 (53)	3 (33)	118 (60)	-	-	-	52 (15)

Values are % of tonic and bursting cells that displayed another activity when hyperpolarised. The total number of tonically active cells are in parentheses.

CHAPTER SEVEN

THE INFLUENCE OF THE LATERAL BASAL LOBE (LBL) ON PCL NEURONS

7.1 Introduction

The lateral basal lobe (LBL) has a vital role in the function of the chromatophore system, providing the only known connection from the higher supraoesophageal regions of the brain to the posterior chromatophore lobe (PCL) (Young, 1971). In some way, the optic lobes translate an external visual cue from the animal's environment to the LBLs, which internally transmit this information to the PCL and anterior chromatophore lobe (ACL), where it is expressed as a pattern on the skin. Morphological studies (Novicki et al, 1990; Young, 1977) and direct electrical stimulation of the LBL (Boycott, 1961) have provided evidence for a connection between the LBL and the PCL. This study, however, is the first of its kind to provide physiological evidence of the effects of the LBL on individual PCL neurons, and also to investigate the activity and morphology of the LBL neurons (for full methods see section 2.4.1).

7.2 Results

7.2.1. Morphology and electrical activity of LBL cells.

The cells of the LBL are very different in morphology from those of the PCL. The LBL cells appeared rather uniform, consisting of a long straight axon and a short and simple array of dendrites with few arborizations. In common with PCL cells are the many swellings of various sizes on the dendrites and axons (Fig. 7.1a). These swellings could be the sites of synaptic contact. The arborizations of all the LBL dyed cells were restricted to the neuropil (Fig. 7.1a & b) presumably because the majority of synaptic connections are made here. The LBL cells were smaller than the PCL cells with a cell soma of approximately 5-10μm in diameter. The majority of the LBL cells, associated with the LBL tract, were situated in the outer lobe of the LBL (Fig. 7.1b). The axons of these cells run downwards towards the tract and into the PCL, where they probably make contact with one or more of the chromatophore motoneurons.

Intracellular recordings were more difficult to obtain from LBL cells due to their small size. Sharper micro-electrodes were used in order to impale the cells, and this made dye injection difficult due to blocking of the electrode. When filled with Lucifer Yellow, the electrodes had a resistance of 100-200Ω, which increased the noise level of the recording. In spite of this, intracellular recordings were made from a dozen LBL cells, and dye was intracellularly injected to reveal their morphologies. Intracellular recordings revealed three different classes of cell in the LBL. The majority of the cells exhibited continuous tonic spontaneous activity (Fig. 7.2). Cells that did not fire tonically were either silent or showed bursting activity (Fig 7.3). Cells that were tonically active displayed EPSPs when hyperpolarised (Fig. 7.2c). Hyperpolarisation also increased the amplitude of the EPSPs and eventually stopped the cell from firing (Fig. 7.2b & c). The EPSPs were partially distorted by the electrode noise due to the high resistance of the dye filled electrode, enhanced by hyperpolarising current injection. In some LBL cells of the same slice large spikes were observed, up to 20mV in amplitude. After electrical recordings, a bursting cell was dye filled and this revealed that the cell soma was located in the outer lobe and that fine dendrites were located along its axon (Fig 7.3). The axon travelled within the LBL neuropil towards the LBL tract and on to the PCL. Various swellings were also seen on the axon and dendrites of this cell.

7.2.2. The LBL to PCL tract.

The LBL to PCL tract was revealed by placing a crystal of the fluorescent marker dye, DiI or DiO, on the tract of paraformaldehyde-fixed slice preparations (for full methods see section 2.6.2). The dye stained the fibres which made contact with the dye crystal, and it was transported some distance along the tract. The LBL tract has been revealed in *Lolliguncula brevis*, using conventional staining techniques (Novicki et al, 1990), but this is the first attempt to use DiI in the squid *Alloteuthis*. Staining of the tract provided a guide for the subsequent physiological experiments.

In stained preparations, the LBL tract could be seen to enter the anterior portion of the PCL (Fig. 7.4a). On entering the PCL, the LBL tract fibres spread throughout the PCL neuropil (Fig. 7.4b). In some preparations, a fibre tract was stained that entered the PCL neuropil. This tract ran dorsally towards the main LBL tract (Fig. 7.4b). This fibre tract did not seem to originate from the LBL and could represent the inter-chromatophore tract which runs between the ACL and PCL. At its origins in the LBL, the LBL tract consists of two main branches made up from finer fibres that converge from the periphery of the lobe (Fig. 7.5). Neurons of the LBL could be seen in the outer areas of the lobe that were connected by fine axons to the main fibres of the tract. The morphology of the LBL tract in *Loligo vulgaris* was very similar to the tract found in *Alloteuthis*, as the tract entered the anterior portion of the PCL and arose from a fairly widespread fibre area within the LBL

In some instances, pallial nerve fibres were stained along with the LBL tract fibres (Fig. 7.6). This could be a result of either accidental staining caused by the spread of dye from the crystal, or these axons could be those of LBL cells. It has been reported that some axons from the LBL cells leave the brain via the pallial nerve directly (Young, 1976).

7.2.3. PCL neuron activity generated by the LBL.

Extracellular field potentials are a measure of the summed activity of many neurons. In this instance, field potential recordings were used to indicate the areas of activity, and the relative magnitude of the activity, within the PCL, evoked by stimulating the LBL tract.

Extracellular field potentials were sampled from the point where the LBL tract enters the PCL to the periphery of the PCL (Fig. 7.7). Field potentials, recorded from the LBL tract itself, revealed a compound rapid potential, probably resulting from the summed spike activity of many axons (Fig. 7.7); the potential here is continuous with the stimulus artefact. Recordings taken further into the PCL, where the LBL tract enters the neuropil area of the PCL, displayed more complex field potentials. Small positively rising peaks were seen immediately after the main peak due to the axon spikes. The timing of this second peak, may

represent the postsynaptic current generated by neurons in contact with LBL fibres; i.e. EPSPs in PCL neurons generated by the activation of LBL fibres (Fig. 7.7). Considering the neuropil area contains the axons of many PCL and LBL neurons, it is not surprising that this complex field potential was observed in the neuropil area. Field potentials recorded at some distance from the site of LBL tract stimulation, in general, showed only single positive peaks that probably represent the direct spread of the LBL fibres into the lobe (Fig. 7.7). From the field potential recordings it would appear that the LBL axons spread throughout the PCL lobe and directly activate at least some of the PCL neurons.

The initial rapid spike component of the field potential was observed at relatively low LBL stimulus strength (2.5 nA) at many points within the PCL, whereas the second positive peak in the field, which represents the point of synaptic connection, appeared only at a higher stimulus strength (3.5 nA), (Fig. 7.8). This is consistent with the view that the lower stimulus strengths were below threshold for generating large postsynaptic responses. The strongest field and the point of synaptic connection between the LBL and PCL neurons could be traced at depth within the slice, by advancing the recording electrode thought the PCL slice at 50 μm intervals (Fig.7.9).

The field potentials were well distributed throughout the PCL and correlated with the spread of LBL fibres within the lobe. There does not appear to be one particular region of the PCL that receives more afferent input than another. The synaptic connections between the LBL cell axons and the PCL cells, shown by complex field potentials, were, however, strongest and most noticeable in the region where the tract enters the PCL neuropil (Fig. 7.7). This was the case for *Loligo vulgaris*, which showed similar field potential profiles to *Alloteuthis* (Fig. 7.10). In *Loligo* however, the extracellular field potentials had additional secondary peaks, perhaps indicating further areas of synaptic connectivity with the PCL cells. Some caution is needed when interpreting what appear to be compound field potentials but which may, in fact, be multiple axonal responses, especially those sampled near the tract (Fig 7.10); clearly the extracellular data should be interpreted together with intracellular recordings.

7.2.4. Effects of LBL tract stimulation on individual PCL neurons.

Intracellular recordings from PCL neurons have provided information on the influence of the LBL tract input on the activity of individual PCL neurons. It was found that not all PCL cells within a slice were affected by stimulation of the LBL tract; many cells, including identified chromatophore motoneurons, did not respond at all. Since the LBL tract is the main input to the PCL this is surprising and the most likely explanation is that some of the connections from LBL tract to these cells were severed during the slicing procedure, or that these cells are only indirectly affected by the LBL tract input.

Electrical stimulation of the LBL tract, at a stimulus strength of between 0.5 and 10 nA, evoked excitatory and inhibitory effects on different PCL neurons. The excitatory input was sometimes an all or nothing event, similar to the antidromic response seen by stimulating the pallial nerve. In these cases, the input from the LBL tract was of sufficient size to cause a spike in the PCL neuron. However, in most cases, stimulation of the tract initially caused an EPSP in LBL cells (Fig. 7.11a) and only upon increasing the stimulus from 0.5 to 1.0 nA, and hence recruiting additional tract fibres, was the input sufficient to cause an action potential in a PCL neuron (Fig. 7.11).

Where a PCL neuron was already tonically active, stimulation of the LBL tract could cause either an increase or a decrease in the level of tonic activity, with excitatory responses being more common than inhibitory responses. An example of the latter is shown in Fig. 7.12. Here, a train of stimuli, at a duration of between 660 to 1700 msec and 8nA stimulus strength, was applied to the LBL tract which resulted in the PCL neuron ceasing to fire during tract stimulation and recovering to its normal firing behaviour when the stimulus was stopped. In some PCL cells, as the duration of the stimulus to the LBL tract was increased, from 660 to 1700 msec stimulus duration, the inhibition increased; action potentials occurred less frequently, and the cell took longer to recover to its previous state. No cell was found to have both an inhibitory and excitatory response to tract stimulation.

7.3 Summary

1) This chapter has set out to for the first time to provide physiological evidence for the influence of the LBL on PCL neurons.

2) Intracellular recordings were obtained from LBL cells that were subsequently dye-filled with LY.

3) LBL neurons have of long straight axons with swellings of various sizes on their dendrites and axons. The cell somata are ~ 5-10 μm in diameter.

4) Intracellular recordings revealed 3 different classes of cells in the LBL:
   A, Cells exhibiting continuous tonic spontaneous activity.
   B, Cells showing bursting activity.
   C, Silent cells.

5) When the LBL cells was hyperpolarised a number of effects were seen:
   A; EPSPs were seen in tonically active cells.
   B; Amplitude of spikes increased in firing cells.
   C: Firing cells stopped firing eventually.

6) The morphology of the LBL to PCL tract was investigated with DiI and DiO. The tract was seen to enter the anterior portion of the PCL.

7) Extracellular field potentials in the PCL, generated by stimulation of the LBL to PCL tract, were measured.

8) Complex field potentials were observed in the neuropil area of the PCL, where the LBL tract enters it.

9) Electrical stimulation of the LBL tract evoked excitatory and inhibitory effects in different individual PCL neurons. Examples are shown.

10) Results suggest that LBL axons spread throughout the PCL lobe and directly activate some of the PCL neurons.

11) These findings have confirmed the LBL connection with the PCL and the significance of this lobe in the control of colour change in the skin.

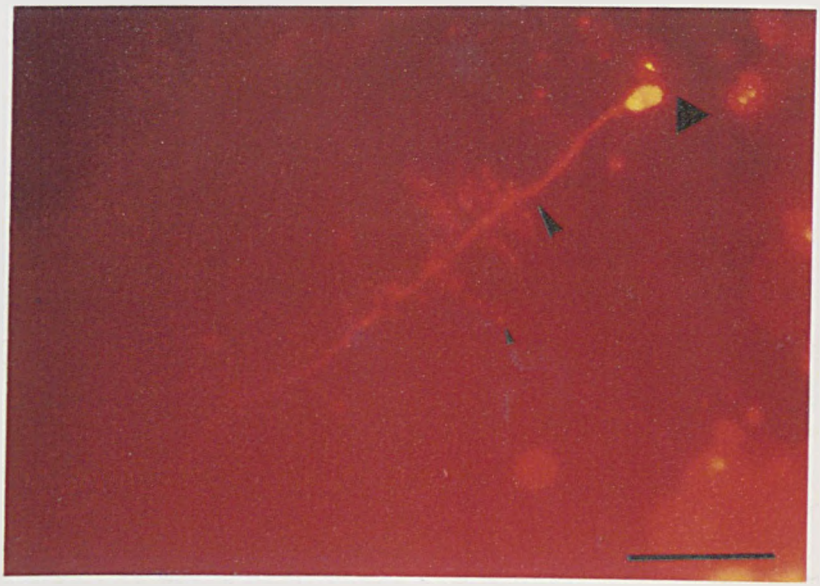


Fig 7.1 (a) DiO filled cell in the LBL of *Alloteuthis* situated on the periphery of the lobe (large arrow head indicates the surface of the lobe/brain). The dendrites of this cell start at the neuropil of the lobe (small arrow) and appear to have swellings similar to those on PCL cells (very small arrows) (Fig. 4.6). The axon travelled ventrally towards the PCL Scale bar: 40 μm.

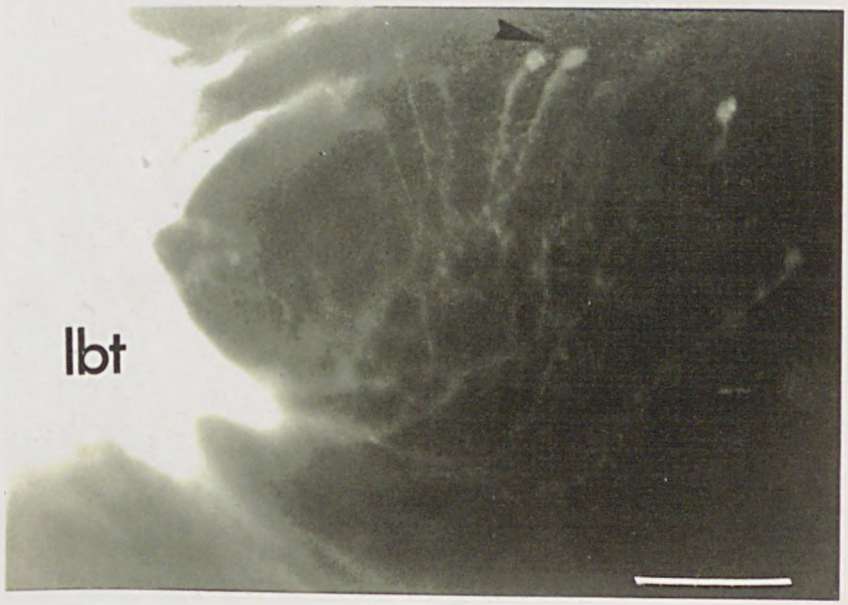


Fig 7.1 (b) DiI filled cells in the LBL of *Alloteuthis*. The neurons were dyed by the placement of a DiI crystal on the LBL tract (lbt). The LBL neurons were located on the periphery of the lobe (arrow) and appeared to have a uniform soma shape and size. Scale bar: 80 μm.

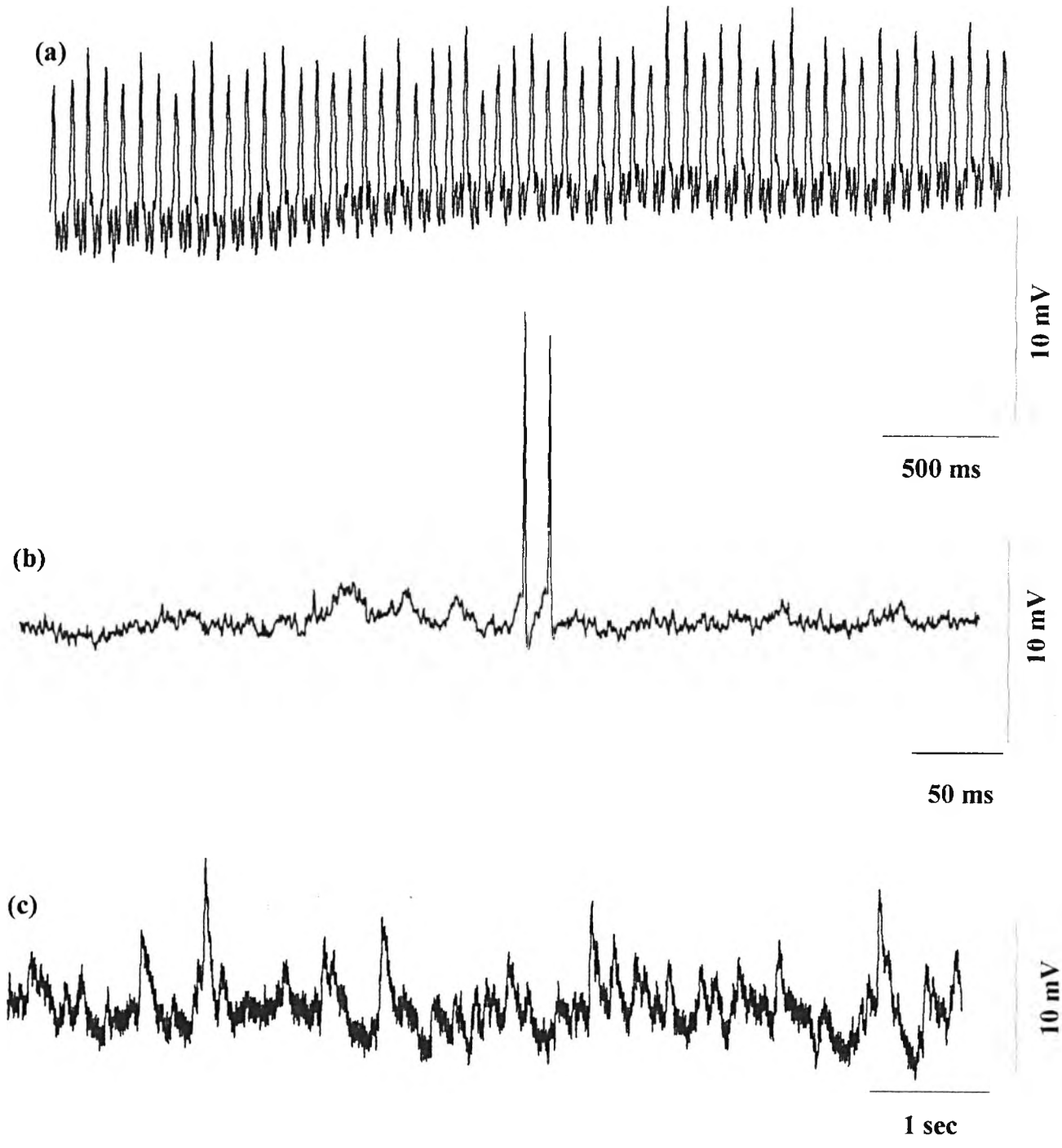


Fig 7.2 Intracellular recordings from a cell in the LBL of *Alloteuthis*. (a) Spontaneously firing cell which had a membrane resting potential (MRP) of -35mV and was located on the periphery of the lobe. (b) The same cell was hyperpolarised to a holding potential of -45 mV, the cell firing rate slowed and the amplitude of the spikes increased. (c) With increased hyperpolarisation to a holding potential of -55mV the noise or synaptic potentials increased.

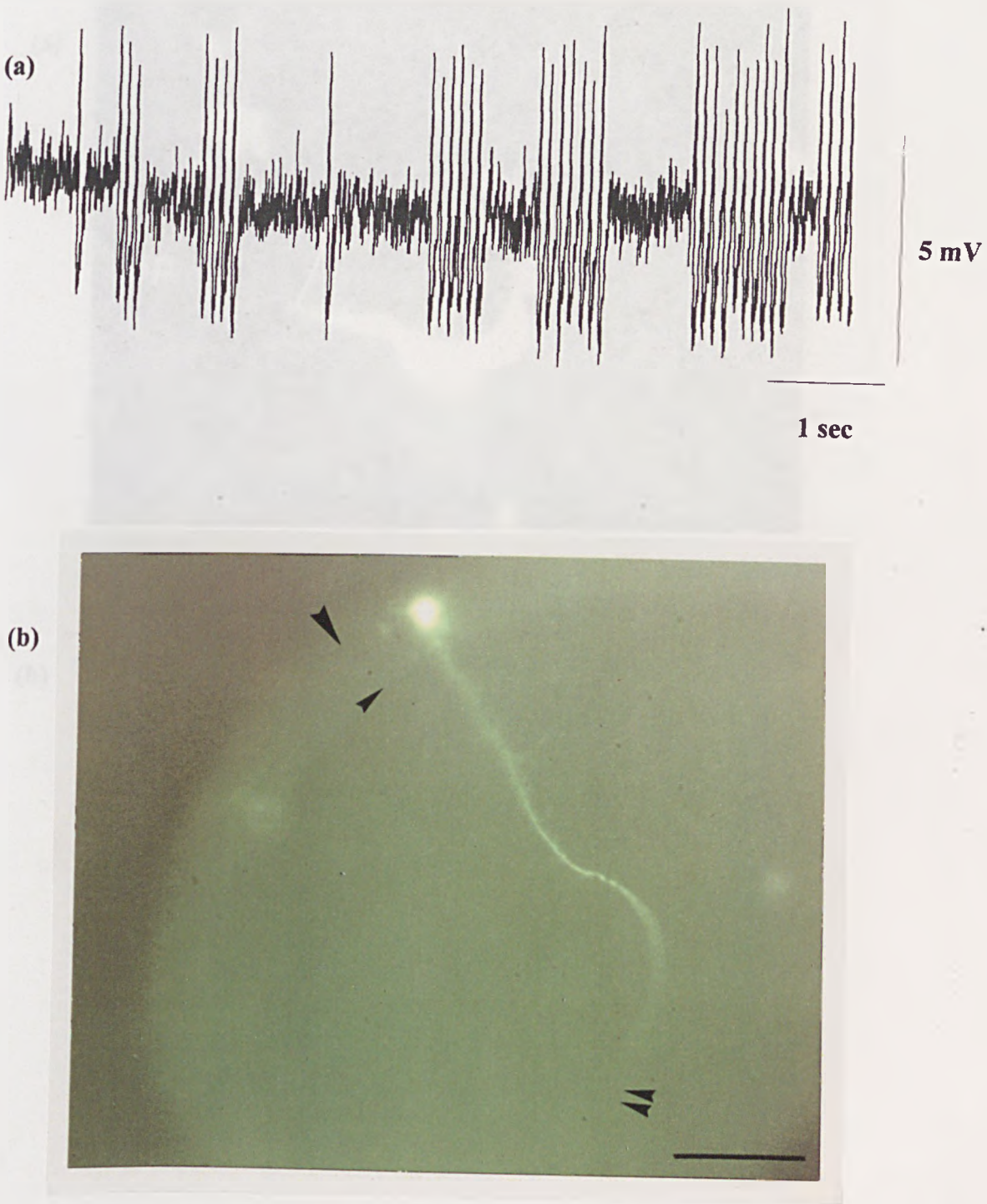


Fig 7.3 (a) Intracellular recording from a cell in the LBL of *Alloteuthis*. This cell displayed bursting activity. (b) The cell was dye filled with Lucifer Yellow. The cell soma was situated in the outer cell layer (large arrow indicates the surface of the lobe, small arrow the neuropil). The axon can be seen to run ventrally towards the PCL (double arrow). Scale bar: 40 μm.

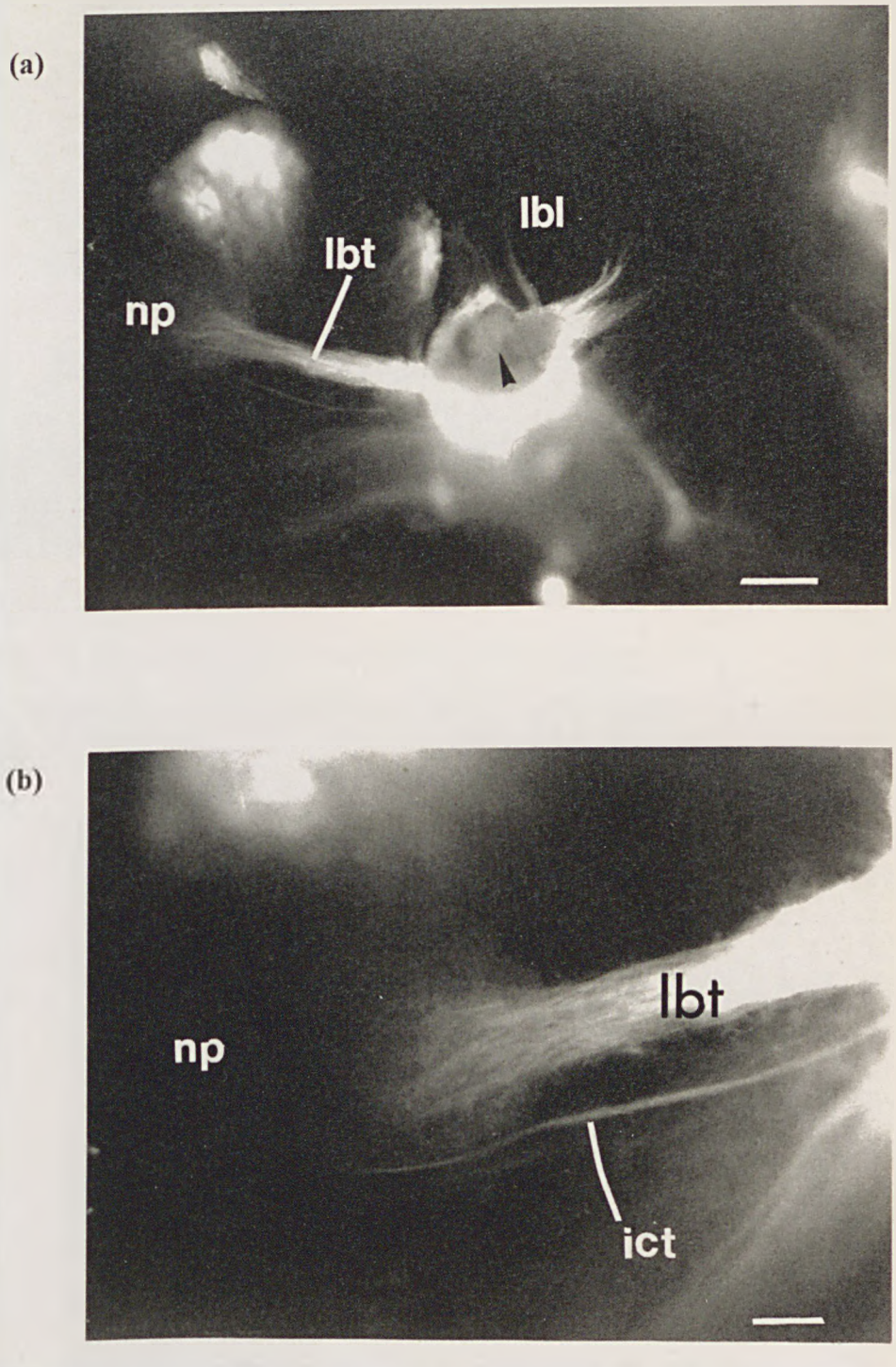


Fig 7.4 (a). The LBL tract of *Alloteuthis* (lbt) enters the anterior portion of the neuropil (np). A DiI crystal was placed on the tract (arrow). Scale bar: 500 μm (b) Higher power view showing the LBL tract (lbt) entering the PCL neuropil (np). The fibre tract can be seen to run dorsally towards the LBL tract, and is the ACL to PCL inter-chromatophore tract (ict). Scale bar: 250 μm.

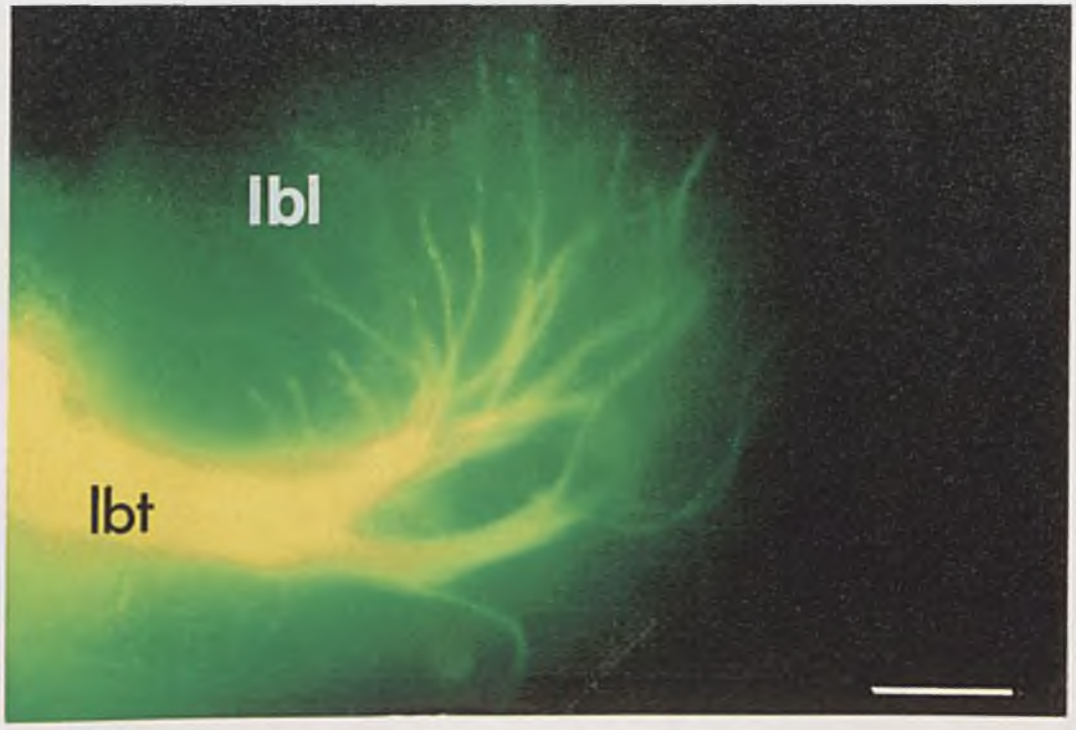


Fig 7.5 DiI filled LBL tract in a fixed *Alloteuthis* PCL slice. At its origin in the LBL, the LBL tract (lbt) arises from two main branches, formed by finer branches and single fibres emanating from neurons on the periphery of the lobe. Scale bar: 80 μm.

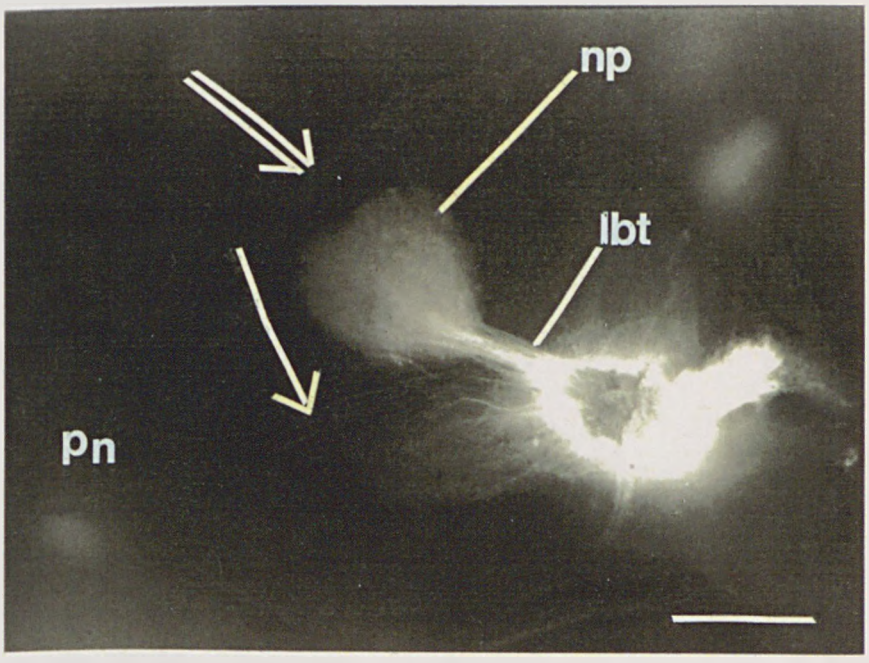


Fig 7.6 DiI filled LBL tract (lbt) in a fixed *Alloteuthis* PCL slice. Some fine axons (single arrow) can be seen to leave the pallial nerve (pn). This picture demonstrates the extensive staining of the neuropil (np). The periphery of the lobe can be seen (double arrow). Scale bar: 500μm

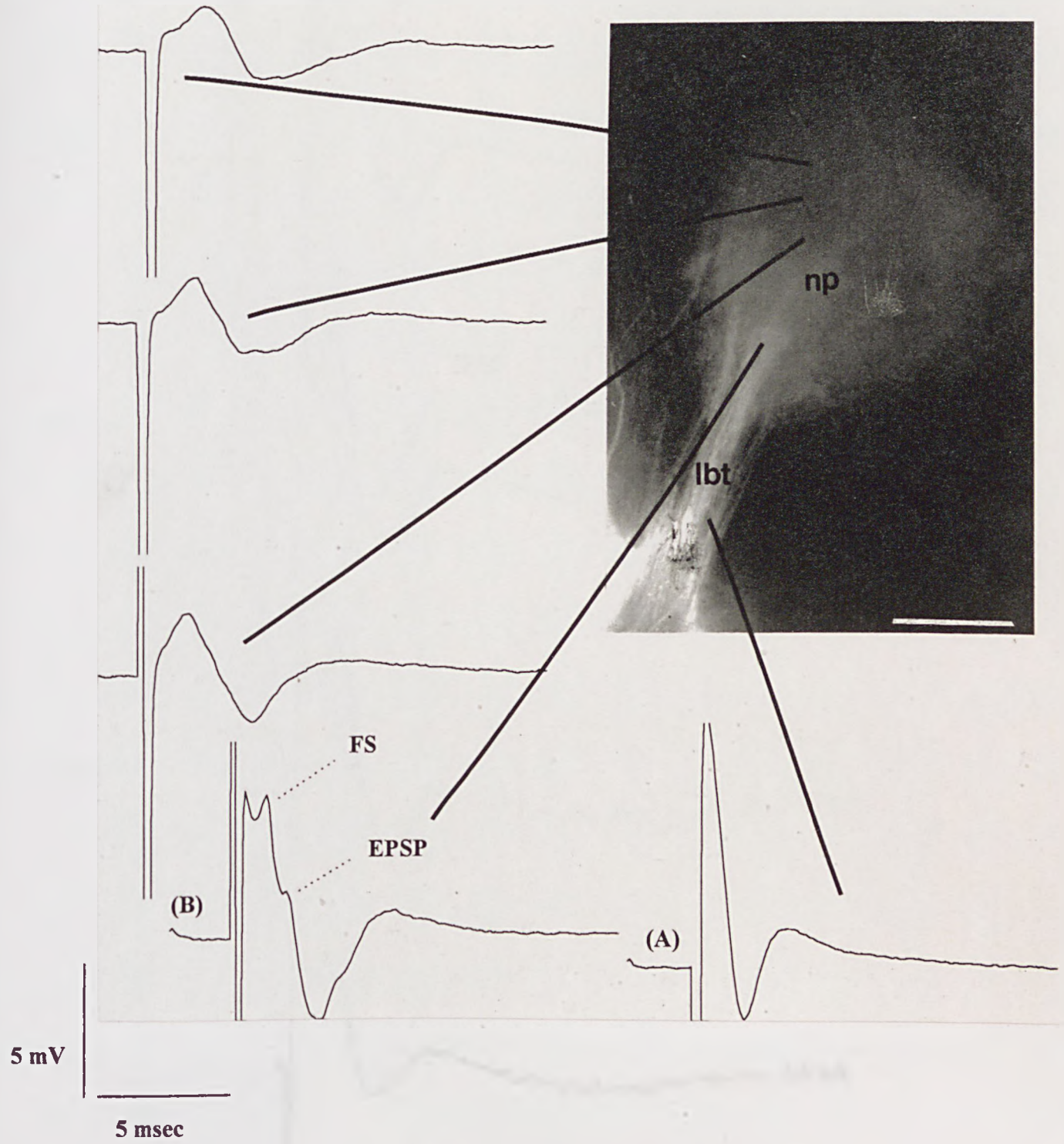


Fig 7.7 Actual field potential recordings from an *Alloteuthis* PCL slice shown in the insert photograph. At position (A), a rapid compound action potential was recorded from the LBL tract (lbt). At position (B) compound potentials with fibre spikes (FS) and EPSP components, were recorded from the PCL neuropil (np). The response became smaller in amplitude towards the periphery of the lobe. Scale bar: 250μm.

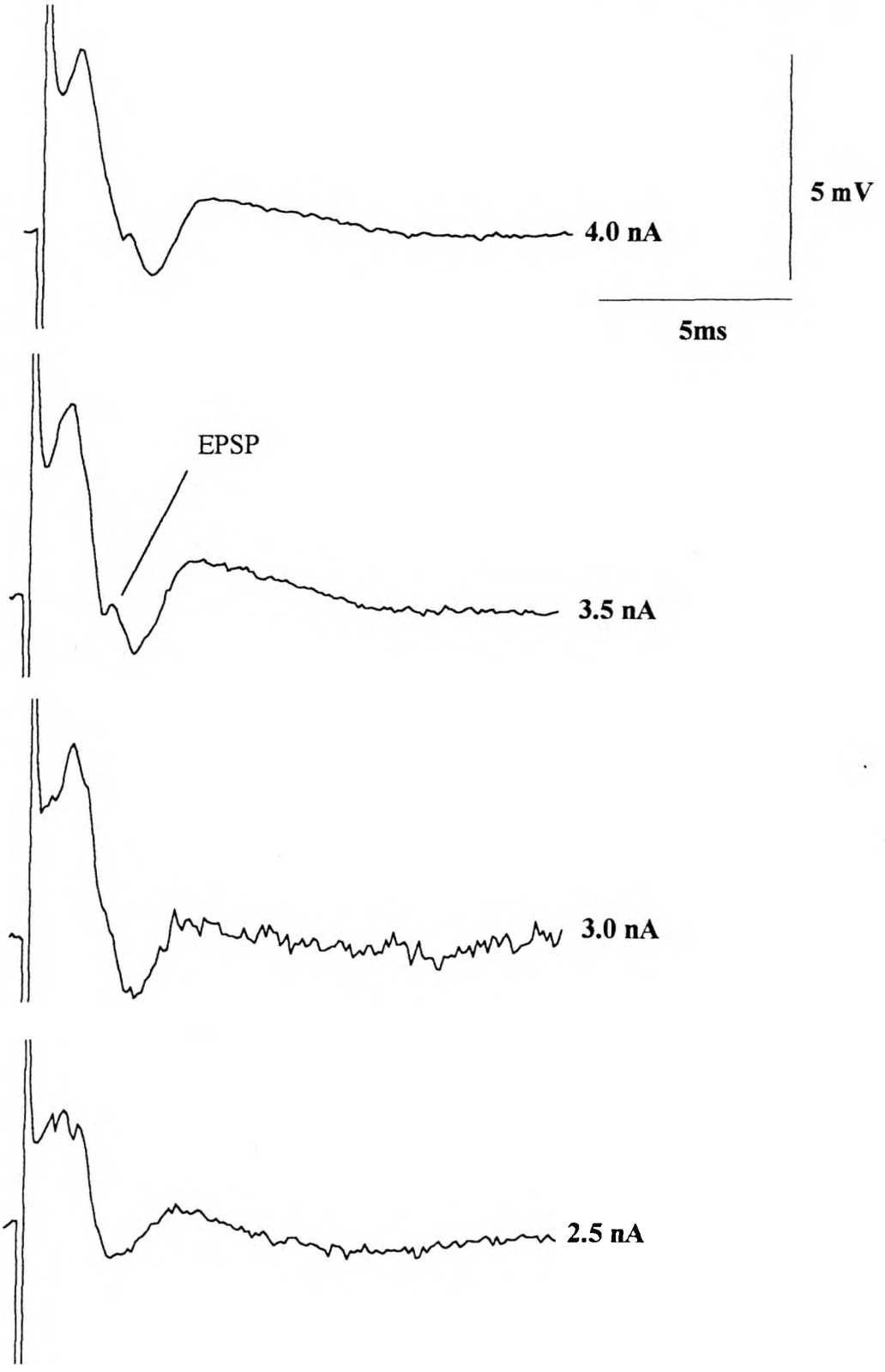


Fig 7.8 The effect of increasing the stimulus strength to the LBL tract, on the field potentials evoked. At 3.5 nA the EPSP component of the field was revealed. Before this level of stimulus there was not a sufficient amount of current to initiate an EPSP response.

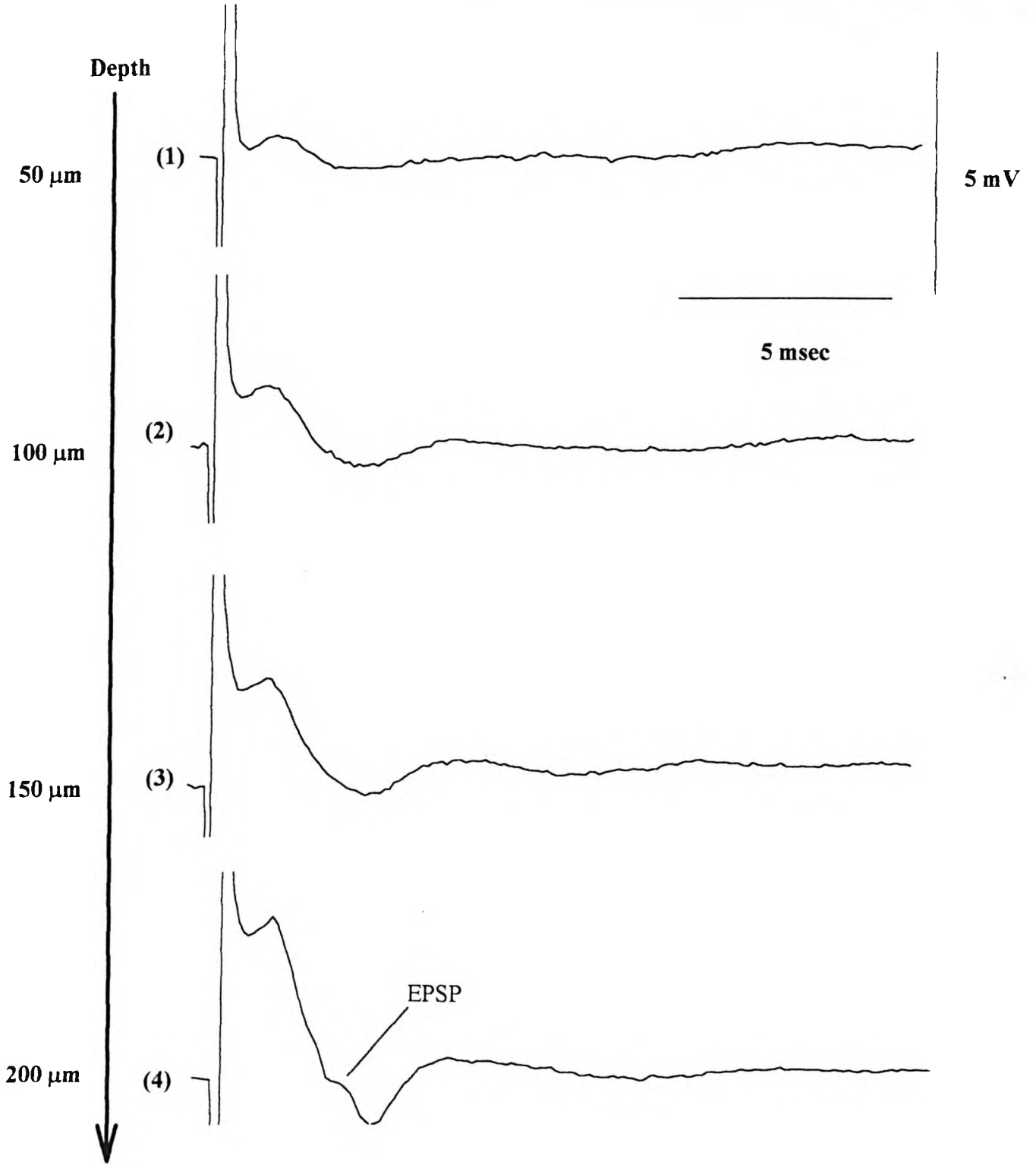
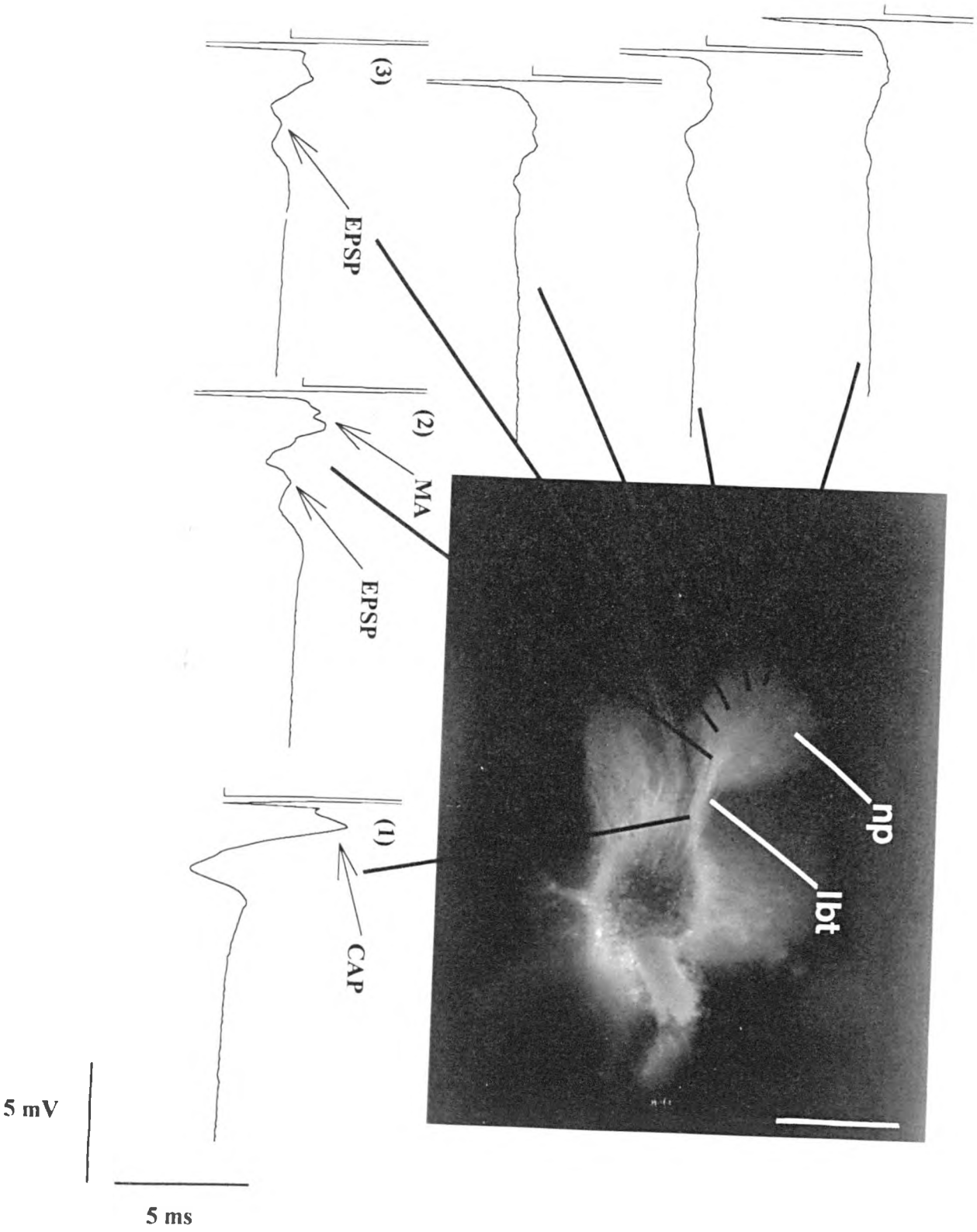


Fig 7.9 The maximum field potentials and synaptic connections were found by inserting the recording electrode at varying depths in the PCL slice. At position (4) a synaptic component could be seen.

Fig 7.10 Field potentials from *Loligo vulgaris*. The recordings were similar to those from *Alloteuthis*, with rapid compound action potentials (CAP) on the LBL tract (lbt) seen in field (1). EPSPs were recorded from the neuropil (np) seen in field (2) and (3), especially where the tract enters the PCL neuropil. The other field potential traces are smaller and less defined than those nearer to the pallial nerve. *Loligo* field potentials were found to be more complicated than those of *Alloteuthis*, with multiple axon effects (MA). Scale bar: 500 μm.


(3)
EPSP
(2)
MA
EPSP
(1)
CAP
np
lbt
5 mV
5 ms

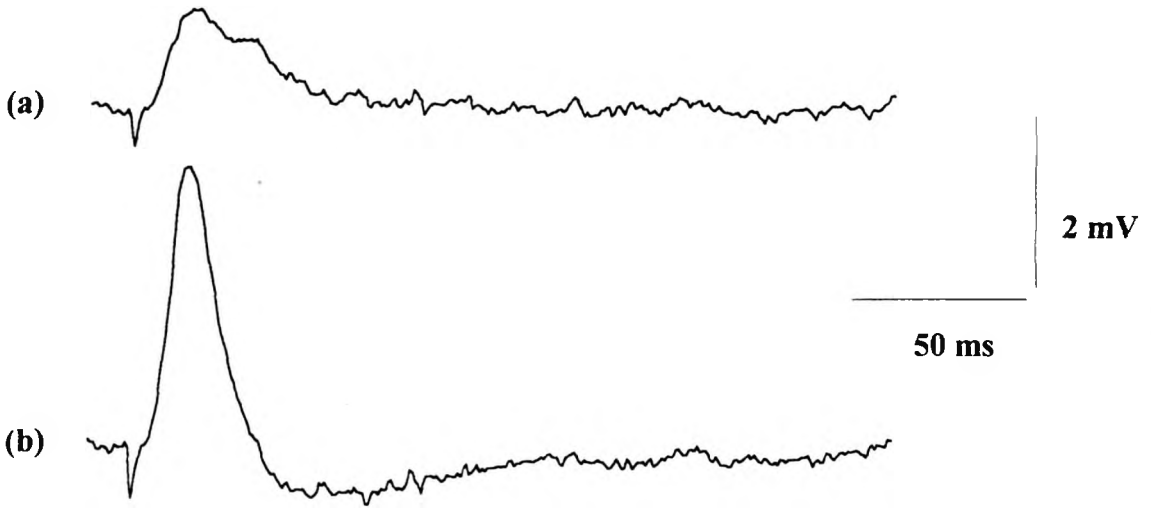


Fig 7.11 Evoked intracellular recording from a PCL neuron in *Alloteuthis*. (a) Shows an initial EPSP response evoked by stimulating the LBL tract at a stimuli of 0.5 nA. (b) Increasing the amount of stimulus, to 1.0 nA in the LBL tract, was sufficient to cause an action potential.

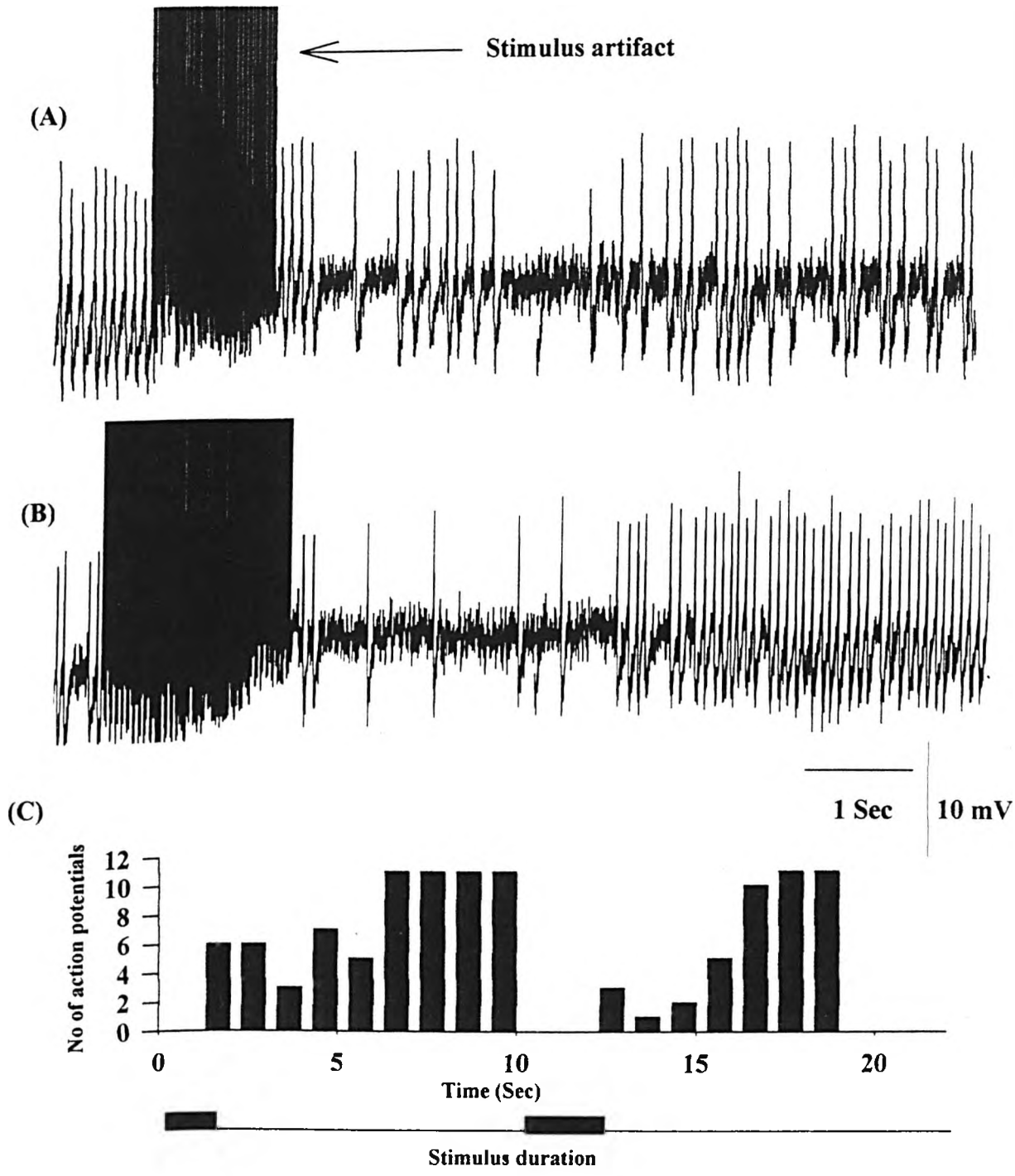


Fig 7.12 Intracellular recording from a spontaneous tonic PCL neuron in *Alloteuthis,* and the inhibition effect from stimulation of the LBL tract. (A) When the LBL tract was stimulated cell firing rate decreased, which can be seen from the trace after the stimulation was stopped. (B) When the duration of the stimulus was increased inhibition of the cell was more apparent. (C) Graph showing the relationship between stimulus duration and the degree of inhibition of spontaneous tonic activity, in the same neuron.

CHAPTER EIGHT
EFFECTS OF NEUROTRANSMITTERS AND ANTAGONISTS ON PCL MOTONEURONS

8.1 Introduction

It has been established that L-glutamate is an excitatory neurotransmitter in the skin and that it causes darkening when applied topically (Bone & Howarth, 1980), and when applied to the brain (Andrews et al, 1983). Subsequently it was shown that this effect could be blocked by the L-glutamate receptor antagonists GAMS and CNQX (Cornwall & Messenger, 1995). 5-HT has been shown to inhibit spontaneous contraction of the chromatophores on the skin, to cause paling when applied topically (Florey, 1966), and to cause interesting mottling effects on the skin when applied to the brain (Andrews et al, 1983). ACh injected into the blood supplying the brain caused the skin to pale, this effect was antagonized by tubocurarine (Andrews et al , 1983).

Despite the detailed and extensive work on the effects of possible neurotransmitters on the skin, there is a lack of work on the effects of neurotransmitters on individual PCL cells. Cornwall & Messenger (1995) suggested L-glutamate and 5-HT are endogenous in chromatophore motoneurons, however, this has not been confirmed. Physiological experiments have demonstrated that there are receptors in the cephalopod CNS which are sensitive to 5-HT (Andrews et al, 1983) but the location of these receptors remains unclear. Brain lesion experiments have implied that 5-HT and ACh act at the level of the suboesophageal lobes to control chromatophores on the skin, but that 5-HT may act at the optic lobe level (Andrews et al. 1983).

In this study, the physiological effects of the possible neurotransmitters on individual chromatophore motoneurons is investigated to clarify; 1) what neurotransmitters are involved at the PCL level 2) what effects they have on PCL motoneuron activity 3) the location and type of receptors present in PCL cells 4) whether 5-HT and L-glutamate are endogenous in the PCL motoneurons and 5) the pharmacology of the connection between the presynaptic LBL cells and the postsynaptic PCL receptors.

Various putative neurotransmitters and antagonists were applied to PCL cells under different conditions; 1) when they displayed spontaneous tonic activity 2) when they displayed evoked responses caused by LBL stimulation. This data represents the first evidence of the effects of applying neurotransmitter antagonists on LBL afferent responses recorded from individual PCL neurons.

The putative antagonist drugs that were applied to the slice were as follows: the L-glutamate non-NMDA receptor antagonists DL-2-amino-4-phosphonobutyric acid (AP-4), gamma-D-glutamylaminomethylsulphonic (GAMS), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), the 5-HT antagonist methysergide maleate, the ACh antagonists tubocurarine (TUB) as a nicotinic receptor blocker, hexamethonium dichloride (HEX) and dihyro-B-erythroidine (DBE) as competitive blockers, and the noradrenaline antagonist, phentolamine (PHEN). The putative excitatory and inhibitory neurotransmitter drugs used were ACh, carbachol and 5-HT. These substances were chosen on the basis of previous published work showing that they had effects on cephalopod nervous tissue (Tansey, 1979; Andrews et al, 1983). Full methods are described in section 2.7.

8.2 Results

8.2.1. The effect of ACh and carbachol on tonic activity in PCL neurons.

When ACh was perfused into the bathing solution surrounding the brain, at concentrations of 10^{-6} M, the frequency of the spontaneous tonic activity of PCL cells decreased until the cells stopped firing. This effect was reversible with washing. The experiment was repeated on three separate occasions for the squid *Alloteuthis subulata*, and produced similar results.

Carbachol was investigated for its effects on muscarinic receptors and had the same effect as ACh. carbachol however is not broken down, as ACh is, by acetylcholinesterase. For these reasons, Carbachol at a concentration of 10^{-4} M was perfused onto tonically active PCL cells; Initially, this caused sporadic activity until after 5 minutes, the cell stopped firing (Fig 8.1).

This evidence is consistent with experiments by other workers, where ACh caused paling of the skin of *Octopus* (Andrews et al, 1983). It is likely that ACh acts as an inhibitory neurotransmitter in some PCL cells.

8.2.2. The effect of 5-HT on tonic activity in PCL neurons.

5-HT when applied via the blood system of Octopus has been shown to cause an interesting mottling effect in the skin (Andrews et al, 1983). In the present experiments 5-HT was applied via bath perfusion to spontaneously active PCL motoneurons in *Alloteuthis subulata.* It was shown in five different cases that 5-HT initially results in an increase in the frequency of spontaneous activity and also hyperpolarised the cell causing a shift in the membrane potential from -35mV to -45mV. The cell finally stopped firing and large EPSPs became more apparent (Fig 8.2). This effect was reversible in three of the five cells after washing with ASW.

These results show that 5-HT had a excitatory effect on the PCL motoneurons being recorded from, and on the presynaptic cell, generating EPSP activity.

8.2.3. Effects of L-glutamate, 5-HT, and ACh antagonists on the LBL tract response.

Extracellularly evoked field potentials and intracellularly evoked synaptic responses in PCL cells were used to observe the effects of bath applied antagonist drugs. Synaptic responses and field potentials, which could be easily manipulated by drug application, were evoked in PCL neurons by stimulating the LBL tract. During drug application, intracellular recordings were frequently lost due to changes in the bath level of the solution which resulted in the dislodgement of the microelectrode from the cell. Extracellular recordings were found to be much more stable than intracellular recordings during solution changes so that the effect of the drug could be observed over long periods of time. For this reason, most of the data on the effects of antagonist drugs was obtained from extracellular recordings of field potentials.

The results obtained are summarised in Table 8.1 & 8.2. Three pharmacological agents, hexamethonium, gallamine, and phentolamine, had no apparent effect on either the amplitude or shape of the evoked field potentials (Table 8.1) even after 45 minutes perfusion and at bath concentrations as high as 10^{-4} $mmol^{-1}$. Of the other agents tested, six, i.e. AP-4, methysergide maleate, GAMS, CNQX, DBE, and high magnesium/ low calcium ASW, were found to have profound effects on the evoked PCL field potentials (Table 8.1). AP-4 and DBE enhanced the responses, while methysergide maleate, GAMS, and CNQX blocked the responses.

The recorded extracellular evoked field potentials were not reversible with washing on some occasions. This is probably because a field potential is the sum of many synaptic responses, some probably poly-synaptic, which are difficult to recover; whereas intracellular responses are probably mono-synaptic responses and therefore more likely to recover with washing, i.e. only one synapse has to recover to regain mono-synaptic responses whereas many have to recover to regain full poly-synaptic responses. Similarly, as with most pharmacological experiments performed on tissue slices, there was some variation in the responses between different preparations. For example, AP-4 had no effect on evoked field potentials on three occasions, but on another three occasions, it enhanced responses, which were reversible with washing. Since each experiment involved stimulation of different groups of axons within the LBL tract, and the field potential was being recorded from different groups of cells, this variation was not unexpected. It does mean, however, that it can be difficult to draw general conclusions from the data. Similarly, Methysergide maleate had no effect on two occasions at quite high concentrations but reversibly blocked evoked responses on another seven occasions, at lower concentrations.

8.2.4. Effects of L-glutamate antagonists on extracellularly evoked field potentials.

The strong extracellularly recorded field potentials obtained from LBL tract stimulation could be partially blocked by CNQX application at a

concentration of 10^{-5} M and 10^{-6} M, this effect however, did not reverse with washing. GAMS, another non-NMDA glutamate antagonist, also reduced and eventually blocked evoked field potentials at concentrations of 10^{-6} to 10^{-4} M, but this effect was also unreversible with washing (Table.8.1 & Fig. 8.5). The irreversible effect of CNQX and GAMS on extracellular field potentials occurred in all the trials (Table. 8.1). The inability of the field potentials to recover, in contrast to the recovery of the intracellular responses (section 8.2.8), is probably a reflection of the number of synapse that are contributing to the response for undoubtedly there will be more synapses involved in the generation of field potentials. Therefore more synapses have to recover after drug application to regain the full poly-synaptic response.

AP-4 showed no effect at concentrations as high as 10^{-4} M (Table 8.1) and enhanced the evoked field potentials at concentrations of 10^{-6} and 10^{-4} M during extracellular recordings of evoked compound field potentials (Table 8.1 & Fig. 8.6). The enhancement of the evoked compound field potentials requires a more complex explanation, as here an additional source of current has been revealed. This current may have been present before the application of AP-4 but may have been previously concealed, possibly due to the summation of another field potential that was blocked by AP-4. Alternatively, an inhibitory input onto these cells, that blocked the additional ion channel and hence, current, was itself blocked by the application of AP-4. In some way AP-4 has changed the synaptic input so as to reveal another current.

The L-glutamate antagonists yielded results with sometimes dramatic effects. CNQX, GAMS, and AP-4 were all shown to block synaptic transmission, with the effects of AP-4 and CNQX being reversible with washing.

8.2.5. Effects of 5-HT antagonists on extracellularly evoked field potentials.

During the bath application of methysergide maleate at a concentration of 10^{-6} $mmol^{-1}$, extracellular field potentials were reversibly reduced in 7 out of 9 trials (Table 8.1 & Fig. 8.7). This drug failed to have any effect in two trials at concentrations as high as 10^{-4} M. Perhaps this failure was due to drug

contamination or insufficient washing from the previous drug, which may have been attached to the postsynaptic receptor sites.

8.2.6. Effects of ACh antagonists on extracellularly evoked field potentials.

Another peripheral nicotinic antagonist DBE, had effects similar to that of the L-glutamate antagonist AP-4, producing an enhancement of extracellular field potentials. Here the depolarising response was decreased and the large negative dip in the field was enhanced. Again, the underlying mechanism is unclear, perhaps a channel was unmasked by the application of DBE. This effect was reversible on washing in ASW.

8.2.7. Effects of Noradrenaline on extracellularly evoked field potentials

The α-adrenergic antagonist phentolamine, which is known to antagonize noradrenergic synapses, had no observable effect on the evoked PCL field potential responses (Table 8.1). This was surprising considering the effects of noradrenaline on the skin of *Octopus* (Andrews et al, 1983). This could be attributed to the failure of the drug itself rather the fact that no Noradrenaline postsynaptic receptors might be present among PCL cells, especially considering that only one trial was attempted (Table 8.1).

8.2.8. Effects of L-glutamate, 5-HT, and ACh antagonists on evoked intracellular recorded synaptic responses from the LBL.

Hexamethonium, gallamine, and phentolamine, had no apparent effect on either the amplitude or shape of the evoked field potentials (Table 8.1) or on synaptic potentials recorded by intracellular penetrations even after 45 minutes perfusion and at bath concentrations as high as 10^{-4} $mmol^{-1}$.

The synaptic responses obtained during intracellular recordings from PCL neurons could be decreased or completely blocked by the bath application of five drugs; high magnesium/ low calcium, methysergide maleate, CNQX, AP-4, and tubocurarine (Table 8.2). These effects on the recorded intracellular responses were all reversible with washing in normal ASW. The L-glutamate non-NMDA receptor antagonist, AP-4 and CNQX, produced reversible blockage of the

intracellularly recorded synaptic responses at concentrations of between 10^{-4} and 10^{-6} M (Table.8.2). With the application of the drugs, the synaptic responses gradually decreased over a period of 15-20 minutes and were recoverable with washing (Fig. 8.4). The 5-HT receptor antagonist, methysergide maleate, reversibly blocked intracellularly recorded evoked responses in two PCL cells (Table 8.2). The cholinergic neuromuscular nicotinic antagonist tubocurarine reversibly blocked (n=4) the evoked responses in intracellular recordings from PCL neurons at concentrations of 10^{-5} M (Table 8.2 & Fig. 8.8). However, the ganglionic nicotinic antagonists hexamethonium and gallamine had no observable effects on the evoked responses (Table 8.1) at bath concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M.

8.2.9. Effects of low $CaCl_2$ and high $MgCl_2$ on evoked intracellularly recorded synaptic responses from the LBL.

As mentioned in Section 6, one test of chemical synaptic transmission is to use a high magnesium/ low calcium solution to reduce calcium entry into the presynaptic terminal and hence block transmitter release. An ASW solution containing 120 $mmol^{-1}$ $MgCl_2$ and 2 $mmol^{-1}$ $CaCl_2$ was perfused onto the slice to investigate if the evoked responses were occurring via a chemical synapse. This solution, when perfused through the bath onto the PCL slice, blocked synaptic responses (n=13), and the effect was reversible on washing with normal ASW (Fig. 8.3). This occurred for both synaptic responses recorded intracellularly (Table.8.2 (n=7)) and for synaptic compound field potentials recorded extracellularly (Table.8.1 (n=6)). Without exception, every observed synaptic input was blocked with the high $MgCl_2$ and low $CaCl_2$ ASW solutions.

8.3 Summary

1) Various putative antagonists were applied to PCL cells under stimulation via the LBL to PCL tract. Various putative excitatory and inhibitory neurotransmitters were investigated in terms of their effects on spontaneous activity of PCL cells.

2) ACh and carbachol decreased spontaneous activity, whereas 5-HT increased the spontaneous activity of these cells.

3) The L-glutamate non-NMDA receptor antagonists, CNQX and GAMS, as well as the 5-HT antagonist methysergide maleate blocked extracellularly recorded field potentials obtained from LBL stimulation. The L-glutamate antagonist AP-4 and the ACh antagonist DBE enhanced evoked field potentials, however.

4) Methysergide, CNQX, and the ACh antagonist tubocurarine blocked intracellularly recorded synaptic responses. AP-4 both blocked and enhanced the response during different experiments.

5) Low $CaCl_2$ and high $MgCl_2$ blocked evoked synaptic responses from the LBL, thereby blocking synaptic transmission.

6) These results indicate that ACh may act as an inhibitory neurotransmitter, upon some cells, while 5-HT may act as an excitatory neurotransmitter in some cells.

7) The implication of these findings is that ACh and 5-HT work together to selectively excite and inhibit chromatophore motoneurons thereby causing selective expansion and contraction of chromatophores on the skin to produce patterning. The fact that 5-HT is present in some PCL and LBL cells suggests that 5-HT affects only certain PCL cells, perhaps pattern generating cells.

8) This work has confirmed for the first time that L-glutamate, 5-HT, and ACh receptors are present on the postsynaptic membrane of PCL cells. It would be advantageous to dye fill those cells that respond to these neurotransmitters, to see if they are of a particular cell type, classified in Chapter Four.

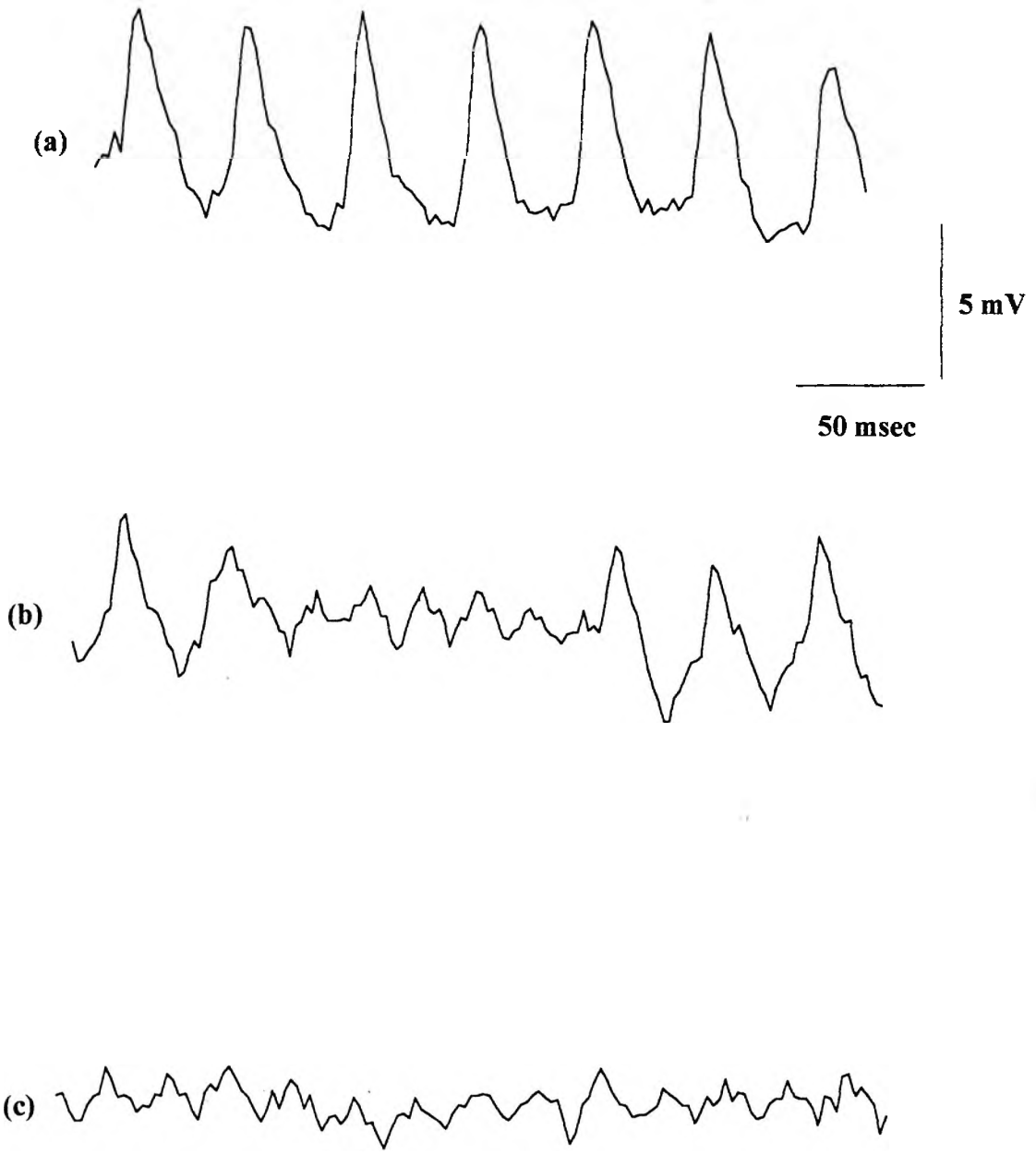


Fig 8.1 Actual traces of spontaneous activity displayed by a PCL motoneuron from *Alloteuthis subulata.* (a) The trace is shown before the addition of drugs (b) application of 10^{-4} M carbachol which resulted in sporadic activity in the cell. (c) Gradually the cell stopped firing and did not recover with washing.

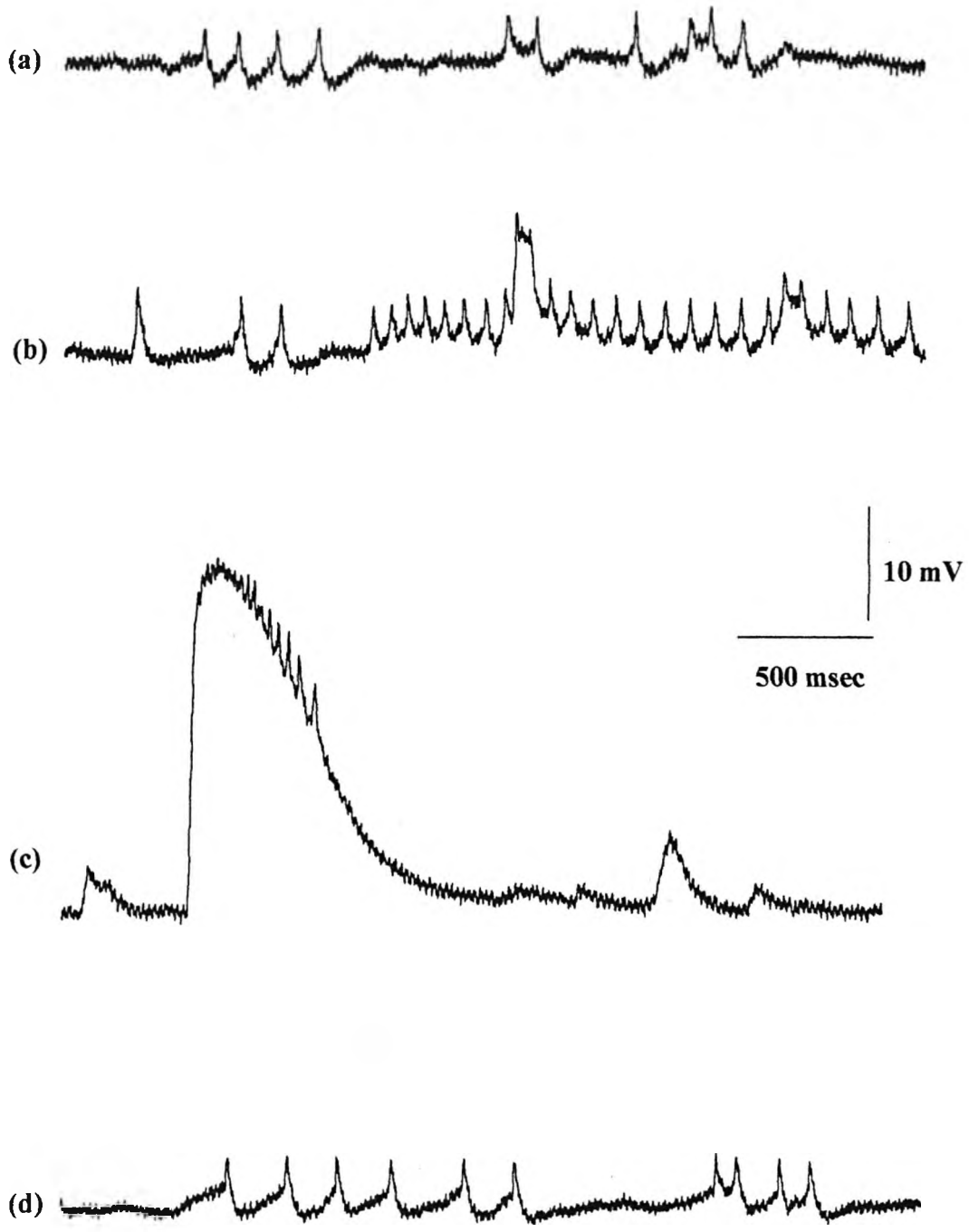


Fig 8.2 (a) Actual traces of spontaneous activity displayed by a PCL motoneuron from *Alloteuthis subulata*. (b) 5-HT at 10^{-5} M was perfused onto the same cell, initially the cell increased its activity in response to 5-HT. (c) With time the cell hyperpolarised and the membrane potential increased by -10mV, the cell stopped firing and large EPSPs were seen. (d) The spontaneous activity recovered with washing.

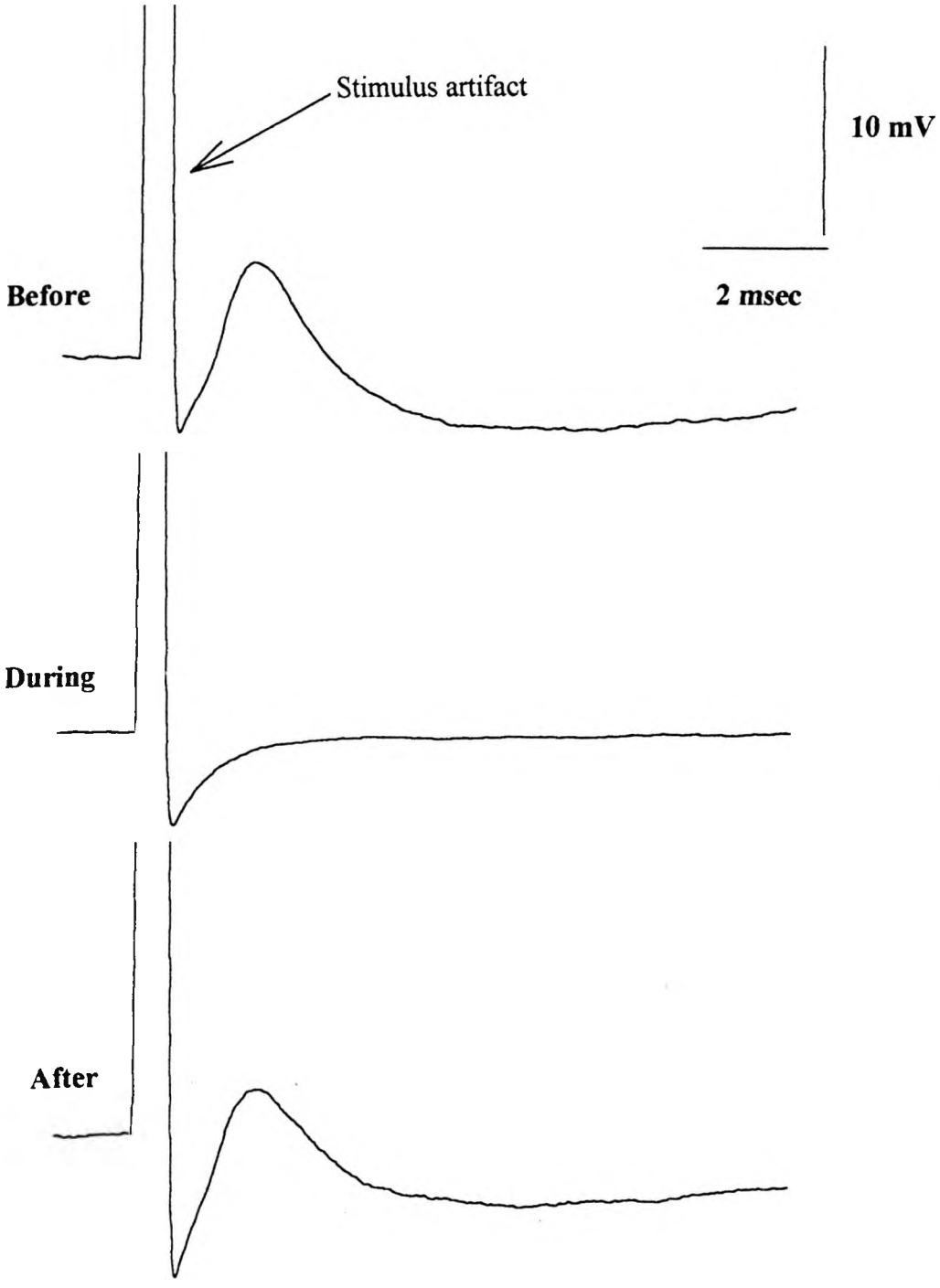


Fig 8.3 An intracellularly recorded synaptic response from an *Alloteuthis subulata* PCL motoneuron, generated by electrical stimulation of the lateral basal tract. The response was recorded before, during, and after the bath application of an ASW, which contained 120 mM $MgCl_2$ and 2 mM $CaCl_2$. The result was a depression of the synaptic effect, during the application of the high $MgCl_2$ and low $CaCl_2$. The synaptic response was recovered with washing with the normal ASW solution.

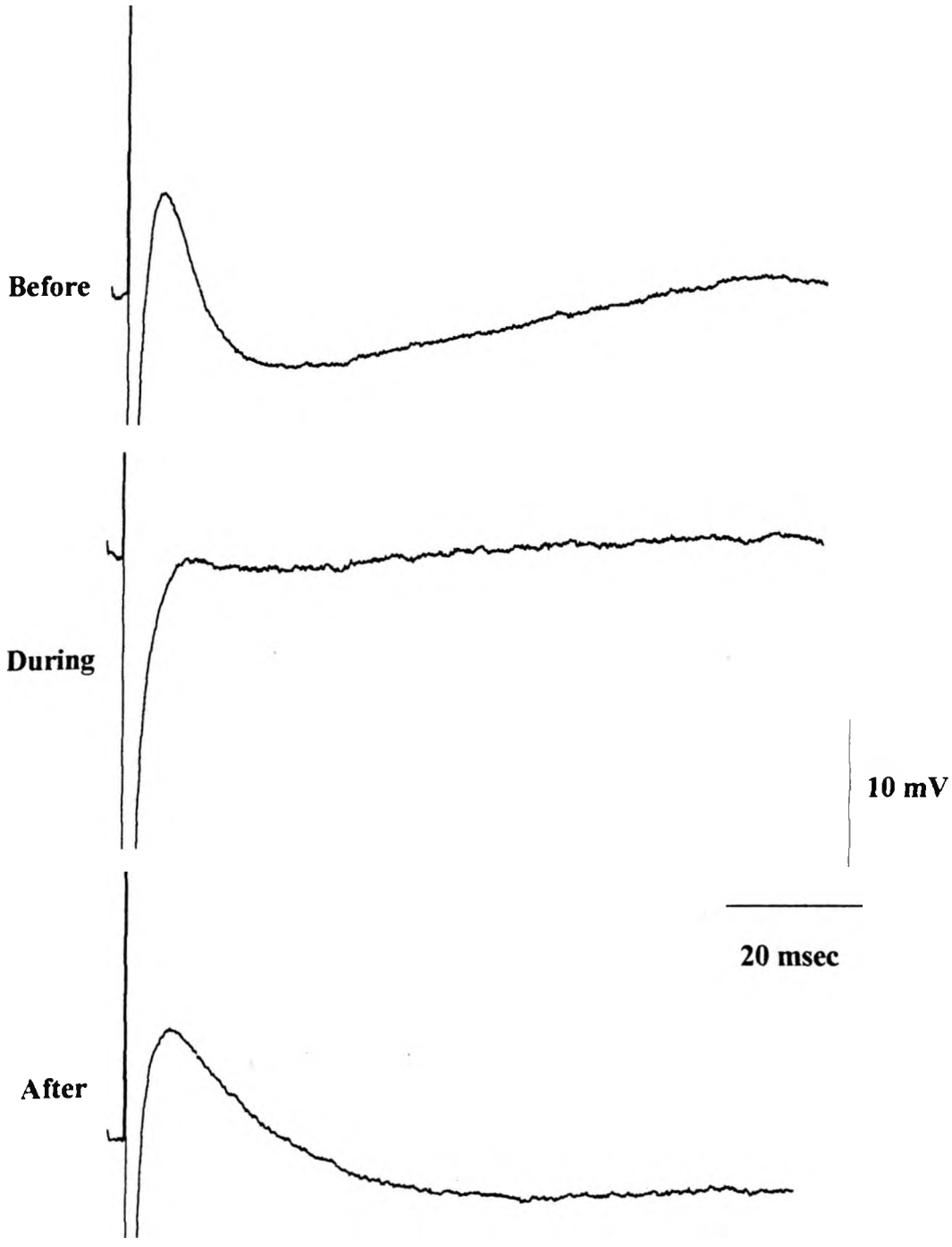


Fig 8.4 An intracellularly recorded synaptic response from an *Alloteuthis subulata* PCL motoneuron, generated by electrical stimulation of the lateral basal tract. The response was recorded before, during, and after the bath application of 10^{-6} M AP-4. The result was a depression of the synaptic effect, during the application of ASW containing 10^{-6} M AP-4. The synaptic response was recovered with washing in normal ASW solution.

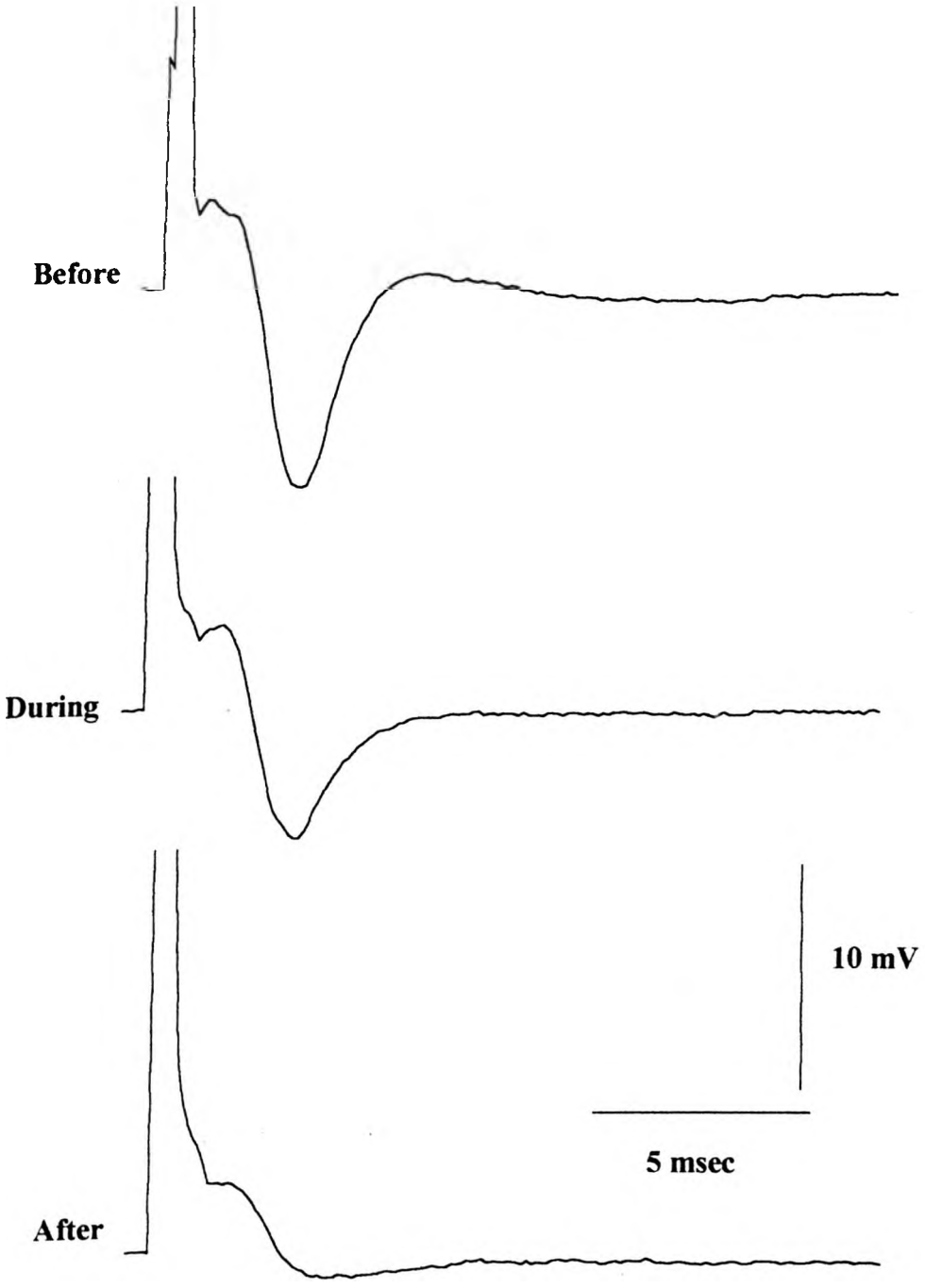


Fig 8.5 An extracellularly recorded field potential response from an *Alloteuthis subulata* PCL motoneuron, generated by electrical stimulation of the lateral basal to PCL tract. The response was recorded before, during, and after the bath application of 10^{-6} M GAMS. The result was a depression of the field potential which did not recover with washing in the normal ASW.

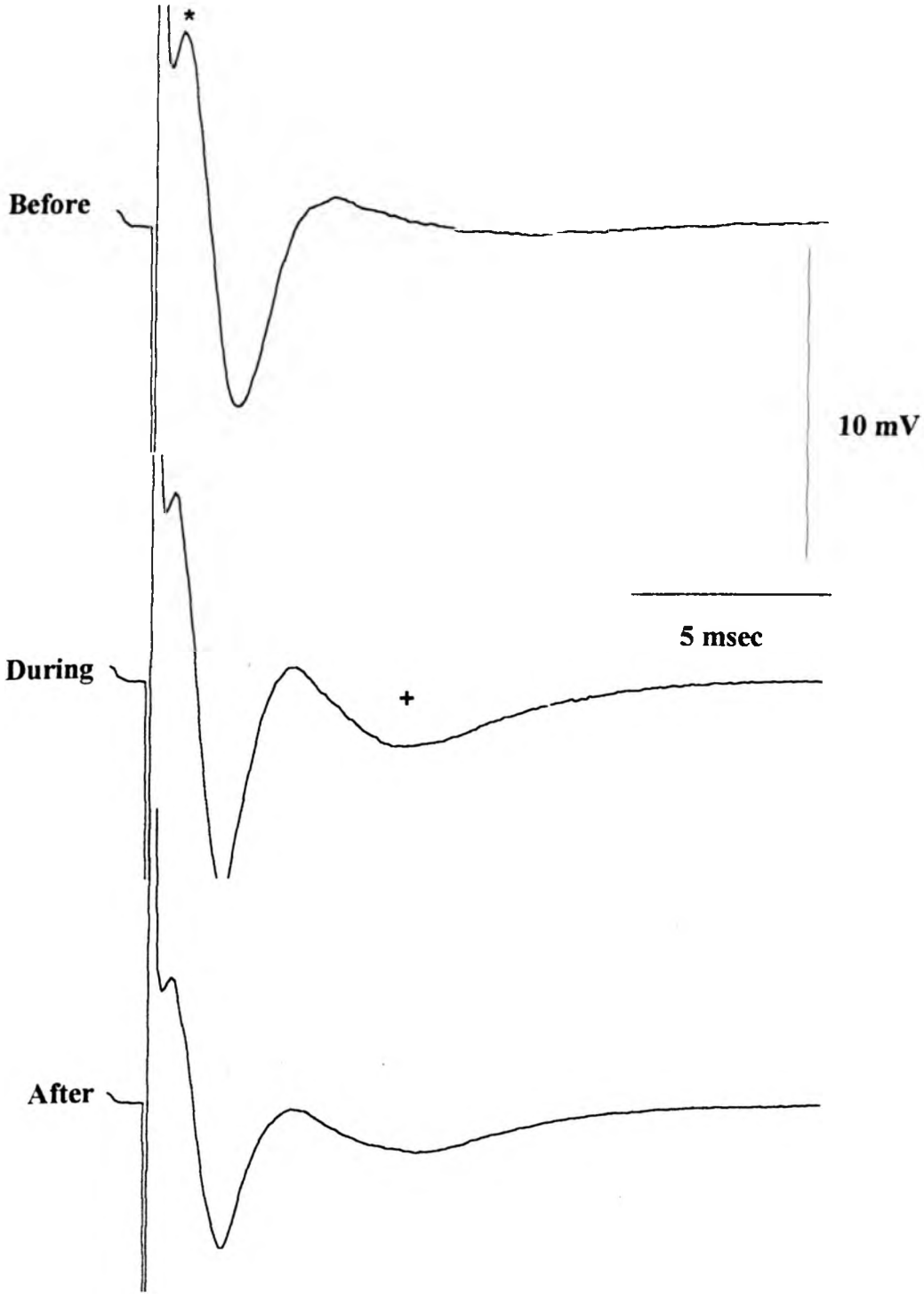


Fig 8.6 An extracellularly recorded synaptic response from an *Alloteuthis subulata* PCL motoneuron, generated by electrical stimulation of the lateral basal tract. In four out of the nine experiments, AP-4 appeared to enhance the field potentials evoked. During the bath application of 10^{-6} M AP-4 the axon spike (*) and the synaptic response (+) were both enhanced. These effects were reversed with washing.

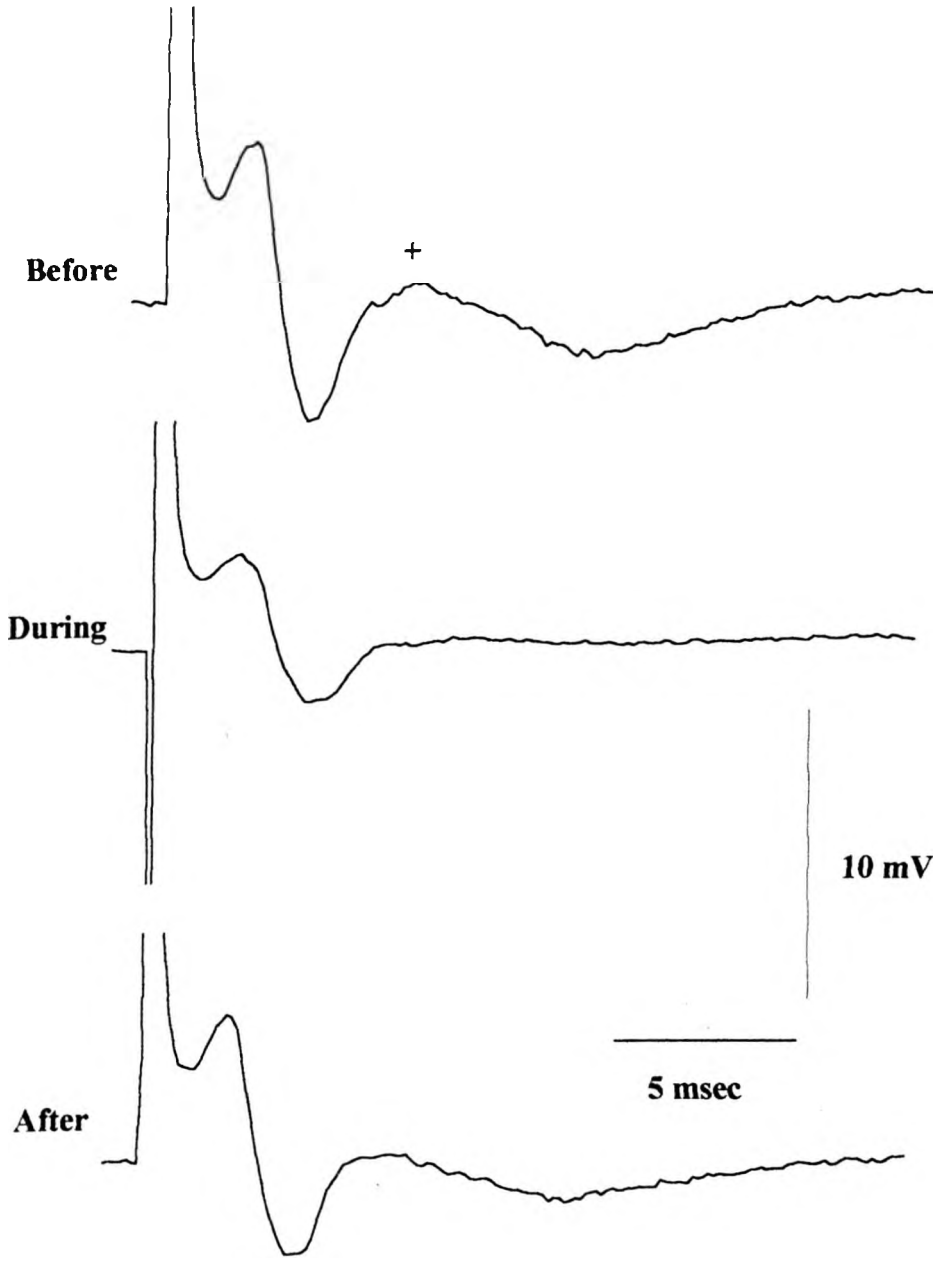


Fig 8.7 Methysergide maleate reversibley blocked extracellular field potential responses, recorded from the PCL. In seven out of seven experiments, Methysergide maleate, at 10^{-6} M concentration, reversibly suppressed the synaptic response (+). In this example the trace began to recover after 20 minutes of washing.

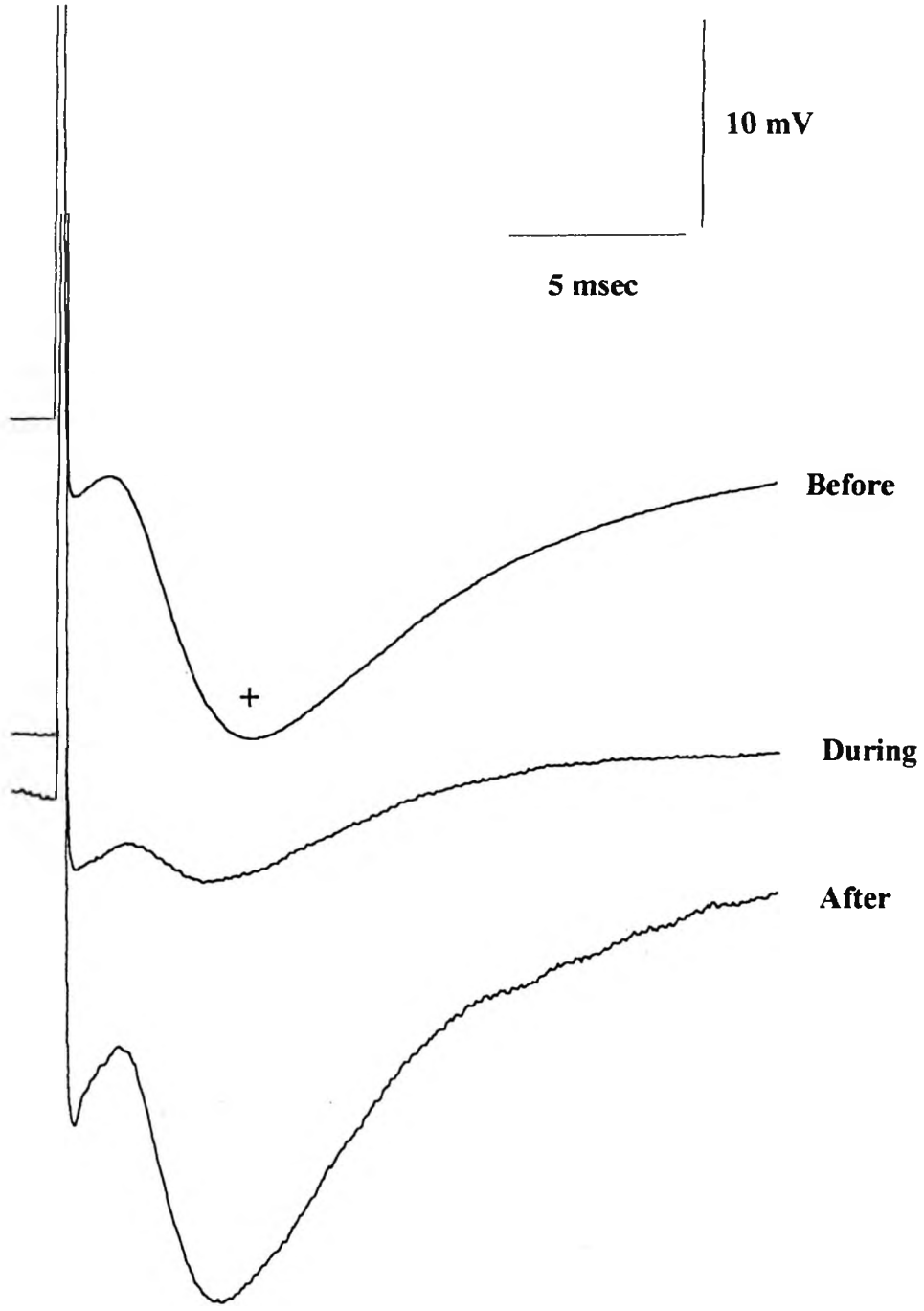


Fig 8.8 Tubocurarine at a concentration of 10^{-5} M had a dramatic suppressive effect on the synaptic responses (+) recorded intracellularly from PCL neurons during LBL tract stimulation. This effect was reversed with washing in normal ASW solution.

Table 8.1 Summary of the effects of certain drugs on the extracellular compound field potentials (CFPs) recorded from PCL neurons of the squid *Alloteuthis subulata.*

Drug	Concentration	No. of experiments	CFP No change	CFP Decrease (Reversible)	CFP Decrease (Irreversible)	CFP Enhanced (Reversible)	CFP Enhanced (Irreversible)
Hex	10^{-6} M	2	2	0	0	0	0
	10^{-5} M	2	2	0	0	0	0
	10^{-4} M	1	1	0	0	0	0
Gall	10^{-4} M	3	3	0	0	0	0
Phen	10^{-6} M	1	1	0	0	0	0
AP-4	10^{-6} M	2	0	0	0	2	0
	10^{-5} M	1	0	0	0	1	0
	10^{-4} M	6	3	0	0	1	2
Methy	10^{-6} M	7	0	7	0	0	0
	10^{-4} M	2	2	0	0	0	0
GAMS	10^{-6} M	1	0	0	1	0	0
	10^{-4} M	2	0	0	2	0	0
CNQX	10^{-6} M	2	0	0	2	0	0
	10^{-5} M	2	0	0	2	0	0
DBE	10^{-4} M	3	0	0	0	3	0
$MgCl_2$ & $CaCl_2$	120mM 2mM	6	0	6	0	0	0

Hex, Hexamethonium dichloride; Gall, Gallamine triethiodide; Phen, Phentolamine mesylate; AP-4, DL-2-Amino-4-Phosphonobutyric acid; Methy, Methysergide maleate; GAME, gamma-D-Glutamylaminomethysulphonic acid; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; DBE, Dihyro-B-Erythroidine.

Table 8.2 Summary of the effects of certain drugs on the intracellular responses recorded from PCL neurons of the squid *Alloteuthis subulata,* during stimulation of the LBL tract.

Drug	Concentration	No of experiments	Synaptic response decreased (Reversible)
$MgCl_2$ & $CaCl_2$	120 mM & 2 mM	7	7
Tubocurarine	10^{-5} M	4	4
AP-4	10^{-6} M	1	1
	10^{-5} M	1	1
	10^{-4} M	1	1
CNQX	10^{-7} M	1	1
	10^{-6} M	2	2
	10^{-5} M	1	1
	10^{-4} M	1	1
Methysergide	10^{-6} M	2	2

DL-2-Amino-4-Phosphonobutyric acid (AP-4), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX).

CHAPTER NINE
DISCUSSION

9.1. Critique of methodology

As with any study, the various techniques employed all have their advantages and disadvantages. The different techniques used in this study and their limitations and advantages have been discussed below with respect to the conclusions made in this thesis.

9.1.1. Animals.

Various animals were used in this study, with the majority of the experiments being carried out on the squid *Alloteuthis subulata.* There was no choice but to use various species because of their availability during certain times of the year.

The majority of experiments were, however, carried out on *Alloteuthis* as this was the most frequently caught species in large numbers during the summer months. It is the smallest of the species, making dissection and brain slicing easier.

During the winter *Alloteuthis* were infrequently caught and when they were the condition of the animals was poor. The majority of winter experiments were therefore performed on the larger and more robust *Loligo vulgaris. Eledone* and *Octopus vulgaris* were used in small numbers for comparison with results obtained by previous studies on brain electrophysiology using these animals.

9.1.2. The brain slice preparation.

The development of a brain slice preparation in this study has enabled new information to be obtained about the PCL. This is mainly because of the limited access to the PCL neurons in whole brain preparations. One of the major advantages of the brain slice, therefore, has been the easy access to individual neurons for morphological and electrophysiological investigations.

The PCL slice has enabled PCL neurons, located near the neuropil and previously never investigated, to be morphologically and electrically identified.

PCL neurons could be classified as chromatophore motoneurons by electrical stimulation of the pallial nerve to evoke an antidromic potential. Neurons in the PCL remained healthy for longer periods of time in the brain slice preparation, compared to the whole brain, due to oxygen diffusion, which was maximised in thin brain tissue. Stable intracellular and extracellular recordings were obtained and this allowed long term perfusion experiments to be carried out. Movement artefacts from respiration and animal movements associated with other previous preparations were eliminated with the brain slice technique. These movement artefacts, seen in previous work have made interpretation of extracellular field potentials (Bullock & Budelmann, 1991) and intracellular recordings sometimes inconclusive (Laverack, 1980). By developing the brain slice the influence of the lateral basal lobe (LBL) on the PCL could be investigated for the first time.

The brain slice preparation, however, is not without its limitations. For example, during the slicing process some damage to cells was inevitable, making morphological and physiological interpretation difficult. The subject of this work, however, was not purely anatomical as the anatomy of the cephalopod brain has been superbly documented in the past (Young, 1971, 1976, 1977) but an intracellular and extracellular investigation of individual PCL cells and their corresponding morphology. This has been considered when interpreting the morphological data, and the results obtained in this work have been compared with those from other studies on the whole brain, to validate the morphological and electrophysiogical results obtained. The thickness of the slice was sufficient to reduce the damage to PCL cells and their connections, but not to compromise the survival of the slice due to oxygen diffusion, which is maximised in thin tissue. This preparation is, of course, not suitable for neuroethological studies.

It was found that the most important factor in producing and maintaining healthy slices was a gentle brain dissection. Taking care not to stretch or compress the brain was essential. The tendency was to perform a quick dissection, which is also critical, at the expense of damaging the brain. The speed of dissection, however, seemed not as important as the care taken in removing and slicing the brain.

Although it is important to acknowledge the limitations when interpreting the data, the brain slice has produced new data and should be ideal, not only for further studies of the chromatophore lobes, but also for studies of other areas of the cephalopod brain and other physiological systems such as those used in memory and learning.

9.1.3. Artificial sea water solutions.

Despite more than 15 years of experience and a wealth of publications (Dingledine, 1984; Jahnsen, 1985), there is no agreement as to the optimal bathing medium for maintenance of brain slices of vertebrates. All of the data on solutions used for cephalopod brain slices, and the likely effects of changing ions in the solutions, are based on comparisons with vertebrate brain tissue slices.

In obtaining an optimal bathing solution and improving the brain slice, brain jelly from *Alloteuthis* was collected and analysed and an ASW medium was developed to mimic this brain jelly. The sample size involved in the collection of the brain jelly was low, due to the difficulty in collecting the jelly which proved time consuming. A larger sample might have produced more reliable and conclusive results. This and other solutions were compared with each other with respect to the quality of field potentials, membrane resting potentials, number of observed dead cells, length of experimental time, and EPSP activity. A single ASW solution was eventually chosen for the majority of experiments, on the criterion that it gave consistent improved results, based on the above comparisons. However, different solutions were used at the beginning of the work and this has made comparisons of the electrical recordings sometimes difficult to compare directly.

An ASW based on a squid tissue culture medium (Rice et al, 1990) was adapted for the cephalopod PCL brain slice and was found to be most effective as a bathing medium.

9.1.4. Dyes and staining.

Although in this investigation, Lucifer Yellow was commonly used as a tracer to demonstrate cell morphology and dye coupling, it does not cross all gap

junctions (Dermertzel & Spray, 1993). Consequently, it may not be optimal for the detection of cell coupling. It has been shown that Biocytin and Neurobiotin, but not Lucifer Yellow, reveal strong cell coupling in the vertebrate retina (Vaney, 1991). Thus, in further studies Biocytin, which has no electrical charge and has a slightly lower molecular mass than Lucifer Yellow, may be a better tracer for studies examining both cell morphology and dye coupling.

Lucifer Yellow, however, has been widely used in this study as it can be injected into the cell by iontophoresis, it is non-toxic, withstands a certain degree of histological processing, and has been used extensively in previous studies. The advantage of employing iontophoresis was that we were able to observe the electrical activity of individual cells and then morphologically identify them with Lucifer Yellow. In this way a comparison could be made between the morphology and electrical activity of the cells examined.

The technique of retrograde transport of dye was used as a method for staining large numbers of cells instead of individual cells. This had the advantage of establishing the areas of likely influence and morphology of the pallial nerve and the LBL tract. However, this method required precise dye application so as to prevent dye spread to other areas in the brain, thus making morphological interpretation difficult.

DiO and DiI proved to be useful and versatile dyes for staining tracts over long distances, such as the LBL tract, and unlike Lucifer Yellow, could be used in fixed tissue so that diffusion of the dye could take place over long periods of time without tissues deteriorating. However DiO and DiI are not readily injected into a cell by iontophoresis and occur in a crystal form. The use of Lucifer Yellow, DiO and DiI was adequate to investigate all the morphological aspects of this work for example: individual cell morphology, dye coupling, and tract and nerve tracing.

It is worth noting that only a small percentage of the total neurons of the PCL has been successfully stained, so that it would be wrong to make definite statements about the whole neural organisation and morphology of every chromatophore cell. Consequently, the morphological data has been interpreted with this limitation in mind and the conclusions are based on differences among the PCL cell morphology rather than on the whole structure of the PCL.

During the slicing procedure, dendrites of some cells may have been severed (as mentioned in section 9.1.2). It could be said that this would account for the differences seen in cell morphology, however the morphological differences between the cell types are too divergent to put down to damage caused by the slicing procedure. Differences that are based on the distance of the dendrite from the cell soma, and the morphology of the PCL cells are comparable to some of the results found in previous studies (Miyan & Messenger, 1995).

9.1.5. Electrophysiology.

The brain slice has provided an excellent preparation to obtain stable intracellular recordings. Individual PCL cells can be viewed in the thin slice, with the advantage of accurate microelelctrode positioning onto a chosen neuron. A number of assumptions were made about PCL cells in this study based on previous investigations. These assumptions include:

1. Chromatophore cells were classified as motoneurons when antidromic spikes were obtained in the cells after pallial nerve stimulation.
2. An intracellular penetration was identified as a sudden drop in the membrane potential, and when no abrupt changes in membrane potential were measured during a recording session.

The use of intracellular recordings allowed individual PCL and LBL cells to be penetrated and the effects of drugs, LBL stimulation, current injection, and electrical coupling to be investigated. These penetrations were made easier by the stable preparation and by being able to view the cell to be penetrated. However, intracellular recordings were not always appropriate for recording changes in the cell physiology with drug perfusion, also intracellular recordings could not be used for monitoring the activity of groups of neurons following nerve or tract stimulation. Hence, extracellular recordings were used to provide a very stable and long term method for recording activity in groups of neurons. This method was particularly useful for investigating the influence of the LBL tract and pallial nerve on the PCL and also in drug perfusion experiments, where the bath volume could sometimes change, resulting in the loss of the intracellular recording. The interpretation of extracellular field potentials, though, is more difficult than

intracellular recordings because the extracellular electrode records the summed current from many sources and these can be very disparate in their amplitude and timings. Also very little previous work has been done in this area, so that comparisons with other work are scarcely possible.

The results gained from stimulating the LBL tract were made possible by the precise method of stimulus applied to the tract using small extracellular monopolar electrodes, thus limiting the current spread and reducing damage to the tract during repeated stimulation. However, damage still occurred to the tract and recordings from PCL cells located some distance from the stimulus were very weak as a consequence. An improvement in the accuracy of the stimuli applied to the LBL tract might reveal stronger response, as a very large stimulus had to be applied to the tract in order to elicit a response in some cells. This consequently caused more damage to the tract fibres and very often led to loss of the field potential response or intracellular response. During this investigation of the sliced PCL, a small part of the LBL tract would have been stimulated and therefore it would be speculative to comment on the entire influence of the LBL on the PCL in this investigation.

The frequency of EPSPs among PCL cells indicates that many of the neural interconnections are still present within the PCL after slicing. The lack of IPSP response does not necessarily indicate that these are fewer in absolute numbers. If the synaptic currents are small, they may not be able to evoke measurable voltage changes in the cell and, in addition, the conditions within the slice may favour excitatory synapses. The number of electrical recordings from PCL cells are low compared to the total number of cells in the PCL. Consequently it would be speculative to make diagnostic statements about the whole neural organisation of the chromatophore system. Nevertheless these results have produced some new data about the chromatophore system that may help future investigators.

9.1.6. Neurotransmitter application.

The effects of certain drugs on evoked extracellular field potentials, intracellular activity and evoked synaptic responses were observed on PCL cells.

Topical applications of drugs to the PCL slice were made with a perfusion system so that the addition and removal of drugs and the washing solution could be controlled in a precise manner, with extended and continuous washes. This type of perfusion was also the most beneficial in maintaining intracellular recordings during solution changes.

Drugs were made up in stock solutions in distilled water and diluted in ASW at pH 7.6 (to match the pH of the normal ASW solution); pH was not therefore a factor determining the reaction of the PCL cells.

9.1.7. Analysis of results.

PCL cells were classified into groups depending on their morphology. In this way a possible relationship was investigated between the morphology and electrical activity of these cells. However some PCL cells did not comply with this classification, and because of the small proportion of PCL cells sampled, compared to the total cell number, it is not possible to make a definitive analysis of the cellular structure of all the PCL.

When a PCL cell was intracellularlly impaled as much data as possible was accumulated from that cell and recorded on a data sheet. The data included: position of the cell, soma size, number of dendrites, distance of dendrites from the cell soma, type of spontaneous activity, evoked responses from the LBL, evoked responses from the pallial nerve, spike amplitude, resting potential, and input resistance. This data could be analysed for later interpretation but cell recordings were often lost before all the data could be sampled and the cell did not always successfully dye fill.

Statistical analyses performed were limited and the results would have been more conclusive if more extensive statistics on a larger sample size had been carried out.

9.2. Review of the main results obtained in the thesis.

The results and achievements obtained from this study can be broadly divided into; the brain slice and sea water solutions, morphology of PCL neurons, evoked response in PCL neurons, intracellular recordings, the lateral basal lobe, and pharmacology.

The brain slice and sea water solutions.

The development of a successful artificial sea water (ASW) solution for maintaining the brain slices during dissection, slicing and long term recording was a major aim of this investigation. During the early stages of the study much time and emphasis was placed on this goal.

This is the first report of its kind to test and compare different ASW solutions on the cephalopod brain slice. The knowledge gained from the effects of changing different solutions, and individual ions, is therefore novel and should be of benefit to other cephalopod workers doing similar research.

The solution which was found to be optimal, was that first used for squid tissue culture experiments (Rice et al, 1990), and surprisingly, the artificial brain jelly solution, consisting of a similar composition to that found in the squid brain, was less good. However, it should be noted that there is some evidence for a blood/brain barrier in cephalopods (Abbott, 1985) and it may be that there are difference between the composition of the brain jelly and that of the brains internal environment. This is hinted at by the observation of Miyan & Messenger (1995) that the chromatophores on the octopus body expanded when a small cut was made in the sheath surrounding the brain.

The results obtained in this investigation showed an improvement over those obtained in *in vivo* studies on the whole brain (Miyan & Messenger,1995; Laverack, 1980), in terms of spike amplitudes, membrane resting potentials, increase length of experimental time (8 hours) and increased spontaneous activity. It is not certain whether the ASW or the brain slice, or indeed both, are responsible for this.

Morphology of PCL neurons.

One of the major advantages of the brain slice is that easy access to individual neurons can be gained throughout the PCL including the neuropil, hence overcoming the limitation of the formerly used whole brain preparation. This has enabled dye filling of over a hundred cells throughout the PCL.

It is clear from this study that squid PCL cells have cell body dendrites as well as axonal processes. This agrees with results obtained for *Octopus* PCL neurons (Young, 1971; Miyan & Messenger, 1995). Young (1971) proposed these cell body processes could form an excitation system, but the function of this is not yet clear.

This study has revealed, for the first time, that the squid PCL is well structured with regularly arranged cell bodies. The largest cells occur at the periphery and the smallest cells occur at the centre. This agrees with what is known about the octopus PCL which has a similar cell body arrangement (Young, 1971). Young (1976) also found that the squid PCL cells are regularly arranged in rows, but no inner layer of smaller cells was noted. It has been suggested that this size arrangement could serve a functional purpose on the skin; the larger cells, on the periphery of the lobe, may innervate chromatophores at further distances away from PCL (Young 1971). Anterior chromatophore lobe (ACL) neurons in *Octopus*, however, are smaller than cells in the PCL despite the fact that they innervate chromatophores on the arms at a greater distances away from the PCL. The size difference of PCL cells could reflect the number of chromatophore muscles that the cell innervates, for chromatophore axons innervate the muscles of several chromatophores (Florey, 1969; Dubas et al, 1986a). Another possible explanation is one of growth; as the animal grows in size so does the skin, hence the number of chromatophores on the skin increases (Packard, 1985). If PCL neurons are added from the inner cell layer, the size difference could be a reflection of the age of the neuron. The bigger and older cells may therefore lie on the periphery, the small newer cells may lie in the inner zones.

On comparing dye fills of PCL neurons, it was found that 83% of PCL neurons could be divided into four different morphological types. This observation was based on the extent of dendrites and the distance of the dendrites

from the cell body. It was also revealed that the different morphological cell types are located mainly in different locations of the PCL. This study is not the first to distinguish different cell types in the PCL. The octopus PCL whole brain preparation revealed categories of PCL neurons similar to those found in this investigation (Miyan & Messenger, 1995) although in that study the sample size was smaller and cells dye-filled were mostly in the periphery of the lobe, because of the nature of the preparation. This is the first investigation to identify the different cell types within particular areas of the PCL cell layer.

Cell Type II was the most common, making up 53% of a total of 95 *Alloteuthis* PCL cells sampled, and was found principally in the outer and middle regions of the PCL. Whether this percentage is a true reflection of that in the PCL is open to debate, as these cells are located in the outer and mid cell layers and had the largest cell bodies and consequently they might be easier to penetrate. This cell type had the most extensive arrangement of dendrites of all. These dendrites were not restricted to the neuropil areas and their processes wandered within the cell layers and between cell bodies. These cells presumably would receive or transmit more synaptic impulses than any other because of their large extensive arborizations. This was confirmed by investigating the electrical behaviour of this cell type. A similar type of cell has been reported in the octopus PCL, but the cell location within the PCL was vague and its electrical activity was not characterised (Miyan & Messenger, 1995).

In contrast, cell type I had a relatively simple morphology with little or no arborizations; this also agrees with findings from the octopus (Miyan & Messenger,1995). This cell type made up only 10% of the total cells sampled, was easily distinguishable from the other cells, and was located principally near the neuropil.

Type III neurons had arborizations that were restricted to the neuropil of the PCL, this type of cell was also distinguished in the octopus (Miyan & Messenger, 1995). Because of the concentration of axons in the neuropil, one would expect the majority of synaptic contacts between PCL cells and LBL cells to occur here. This assumption was confirmed by the electrical data collected from this cell type.

Type IV neurons were located mainly in the inner cell layers with the cell soma being closely associated with the neuropil. These cells had soma dendrites that were at times as thick as the axon. These soma dendrites could be used for short range signalling to other PCL cells; morphological evidence of some joining of soma dendrites to other PCL cells suggests that this could be the case. Cell type IV was seldom dyed filled, perhaps due to its small size and location, rather than the low numbers of this cell type.

Clearly the PCL of the squid is complex, and the cells are far from homogenous, with distinct morphological cell types located in different regions of the lobe. Not only do they look different but they also behave different electrically. It is clear, on comparing octopus and squid PCL cell structures, that similarities exist between the species, despite the very different life styles of the two animals (Boyle, 1983). This indicates that the morphological arrangement of the PCL cells is significant in some way in controlling the chromatophores on the skin. It is worth noting, however, that only a small percentage of the total neurons of the PCL has been successfully stained, therefore, it is premature to make definite statements about the whole neural organisation and morphology of every chromatophore cell. During the slicing procedure, dendrites of some cells were severed and it could be argued that this accounts for the differences in observed cell morphologies. However, the morphological differences between the cell types are too divergent to be attributed to damage caused by the slicing procedure, and the morphological results from this investigation largely confirm a previous study on the octopus whole brain preparation (Miyan & Messenger, 1995).

Not all PCL cells conformed to the four cell type classifications discussed above, 17% of PCL cells did not conform to this categorisation. Cells with short axons that did not leave the PCL were also seen; these are likely to be interneurons. These could be involved in short range communication between other PCL cells. An intracortical with no processes leaving the cell layer has been seen in the octopus (Miyan & Messenger, 1995), but interneurons or short axon cells, have not been reported before in the squid (Young, 1976).

Observations of the detailed anatomy of the PCL cells revealed swellings of various sizes at the ends on the dendrites or elsewhere along the dendrites; these may represent boutons and agrees with the findings of Miyan & Messenger (1995).

Dye coupling was found to exist between 5% of neighbouring squid PCL cells. This finding confirms the findings for electrical coupling in the present study and is also comparable with the results of dye coupling in octopus (Miyan & Messenger, 1995). This is the first study to investigate and reveal electrical coupling in PCL cells, although this had been proposed for octopus PCL cells on the basis of dye coupling by Miyan & Messenger (1995). Some dyed filled cells did not show signs of electrical coupling. But electrical coupling and dye coupling, often considered in parallel, can occur independently (Audesirk et al, 1982). Hence, even though dye coupling was not observed in many PCL cells, it does not mean that electrical coupling does not exist in these cells. Electrical coupling is one mechanism which may co-ordinate the simultaneous activation of different chromatophore motoneurons and therefore groups of chromatophores on the skin.

Evoked response in PCL neurons.

Responses in PCL cells were evoked in two ways; 1) by stimulation of the pallial nerve and 2) by the stimulation of the LBL tract. The evoked responses were recorded both intracellularly and extracellularly in PCL cells.

An antidromic spike was evoked in the majority of PCL cells, and based on the findings of others, which revealed that the pallial nerve travels to the skin and innervates the chromatophore muscle (Sereni & Young,1932; Dubas et al 1986b), the cell was identified as chromatophore motoneuron. The latencies of the evoked antidromic spikes varied in different cells according to the position of the cell within the PCL. Generally the further the cell was located from the point of stimulation of the pallial nerve, the longer the latency of the spike generated. This can be attributed to the conduction time of the spike travelling along the axon.

This study has shown for the first time that chromatophore motoneurons can be electrically identified. These results correlate well with previous studies which identified chromatophore motoneurons morphologically (Dubas et al, 1986 a&b) and it is clear from this study that the brain slice is a viable preparation for preserving PCL cell axons. This novel finding should enable future researchers to investigate the effects of stimulating identified chromatophore motoneurons on the skin.

Evoked extracellular field potentials were recorded from the cells of the PCL, these were widespread but distinctive. This is the first account to document field potentials from the PCL. These responses were comparable to those found in the intact octopus brain, however, in those experiments the location from which the field was recorded was inconclusive (Bullock, 1984). The distribution and amplitude of the evoked field potentials correlated well with the data obtained on the morphology of the pallial nerve fibres throughout the PCL, from this study and from other studies (Novicki et al, 1990). Field potentials with the largest amplitudes, and consequently regions of most activity, could be directly related to the distribution of the pallial nerve fibres. This data has identified specific areas of high electrical activity generated in chromatophore motoneurons.

Intracellular recordings from PCL neurons.

During this study over three hundred intracellular recordings were obtained from PCL cells, over a hundred of which were dyed filled subsequently. The results obtained from the electrical recordings are comparable with those obtained in other cephalopods (Miyan & Messenger, 1995 Laverack, 1980, Bullock & Budelmann, 1991).

There are a number of findings that are noteworthy and which merit detailed examination. First, the amount of recorded evoked and spontaneous excitatory postsynaptic potentials (EPSPs) is interesting. It was found that cell type III displayed more EPSP activity than any other cell type. This finding is consistent with the fact that these cells had dendrites restricted to the neuropil and that the majority of synaptic activity was recorded from this area. This result,

together with other data collected in this study and in other studies on coupling between PCL cells, indicates that there is a complex level of communication and integration in the PCL. Unfortunately, the functional significance of this level of EPSP activity in colour production on the skin of the animal is still beyond a full explanation. However, what is significant and certain from this result is that the brain slice preparation keeps the neural connection of PCL cells intact.

The second interesting phenomenon was the distinctive and noticeable regular bursting activity seen. Such activity was seen in cell type II only. A similar type of activity was recorded in the whole octopus brain and was believed to be in the region of the pallio-visceral lobe (Laverack, 1980). The importance of this electrical behaviour on the skin is unclear without further investigation, but perhaps the pulsating behaviour of the chromatophores on the skin of cephalopods can be attributed to the regular bursting activity of cell type II, either directly or indirectly. However, an earlier study on *Aplysia* pointed out that bursting in cell R15 occurs only in a totally isolated preparation such as the one used here (Stinnakre & Tauc, 1969). Further investigation into the significance of this type of electrical activity in a live preparation would need to be obtained to clarify this point.

The third interesting phenomenon was the spontaneity of discharge shown in tonically active PCL cells. Tonic activity was the most common activity seen among all the cells in the PCL, particularly cell type I and IV in which it constituted 70% of total cell activity. The frequency of this tonic activity varied between different cells, with firing frequencies of between 1 and 12 Hz. This finding agrees with the fact (1) that in isolated squid skin the chromatophores show maximum expansion when stimulated at 12-15 Hz (Florey, 1966) and (2) that similar events have been reported for octopus whole brain preparations (Miyan & Messenger, 1995). It is proposed that the different frequencies of the spontaneous tonic activity could control different degrees of chromatophore expansion on the skin, and possibly the number of chromatophores innervated, which would provide a fine control of colour change, for example during countershading in squids (Ferguson & Messenger, 1991).

The variability of PCL cell resting potentials, which ranged from -20mV to -60mV, could indicate possible differences in groups of neurons. However, no significant division was observed in the resulting potentials between the different cell types characterised. The range of resting potentials seen is consistent with those observed in other studies (Williamson & Budelmann 1991; Laverack, 1980; Miyan & Messenger,1995). Intracellular penetration of PCL cells that had large resting potentials of -60 mV showed these cells to be generally inactive, with no signs of synaptic activity, and invariably an unresponsiveness to intracellular depolarisation. On the other hand, cells with lower resting potentials were generally active or could be excited by external or internal stimulation. A glial origin for the non-excitable cells is possible but in view of the considerable number of such cells encountered and the comparative scarcity of glial (Laverack, 1980), this explanation seems unlikely.

The amplitude of spikes recorded from PCL cells varied and ranged from 2 mV to 40 mV. The recorded spikes were larger than those obtained from similar studies (Miyan & Messenger, 1995) and this possibly reflects the improvement of access to the PCL cells, which enabled recordings to be made from the cell axons. The variation in spike amplitude is quite possibly due to the location of the recording electrode in the cell It has been implied in octopus that the cell bodies are electrically inactive and spikes are initiated at some distance from the cell body (Laverack, 1980; Williamson & Budelmann, 1991). This would account for the variation in the spike recordings seen.

Electrical coupling was observed between neighbouring PCL cells. The result was that the two cells were synchronised in their activity. One cell was identified as a chromatophore motoneuron and both displayed tonic activity. This finding was confirmed by dye coupling which was revealed in these and other cells. This is the first report to investigate electrical coupling in the PCL neurons and consequently comparisons are impossible with other preparations or cephalopods. The influence and significance of this finding is unclear because it has not been extensively investigated and the numbers of cells sampled was very low. Such coupling may be used in the rapid communication between PCL cells

and is possibly essential for co-ordinating the activity of a population of cells (chromatophores) on the skin.

This study is the first of its kind to attempt to record the electrical activity of PCL cells and to relate the cells electrical activity to its morphology, in doing so revealing that the distinct morphology cell types behave electrically different.

The lateral basal lobe.

One of the most interesting findings of this investigation has been the discovery of the inhibitory and excitatory influence of the LBL on PCL neurons. This is the first report to reveal a physiological link between the LBL and PCL and confirms earlier morphological investigations of this connection (Novicki et al, 1990; Dubas et al, 1986b). The morphological data obtained in this study confirms that of earlier work which showed that the afferent fibres from the LBL are evenly distributed throughout the neuropil of the PCL (Novicki et al, 1990). The afferent responses obtained from PCL cells, generated by electrical stimulation of the LBL to PCL tract, were also evenly distributed.

From the intracellular recordings it was clear that both excitatory and inhibitory responses were evoked in different cells. Some cells showed no responses at all. Because a limited number of LBL axons are stimulated in the slice preparation, compared to the whole tract in the intact animal, it would be inaccurate to suggest that the cells with no responses receive no input from the LBL. The excitatory and inhibitory responses shown in PCL neurons could provide some clues as to the chromatophore behaviour on the skin of the animal. The LBL may exert its effects on the chromatophore motoneurons by direct contact onto the PCL cells and/or by indirect contact via PCL interneurons that have an inhibitory or excitatory synapses onto the chromatophore motoneurons.

The differences in the synaptic delay of the evoked EPSPs generated in PCL neurons by stimulating the LBL tract, may indicate that polysynaptic connections are made from the LBL cells onto PCL cells. Such inputs could originate from two or more synaptic connections from the same LBL cell, or from two different LBL cells, each with a synapse onto the PCL cell, or via PCL interneurons. Assuming that in *Alloteuthis* there are more PCL neurons than LBL

neurons (in octopus there are twice as many cells in the PCL than in the LBL (Young, 1971)), and that the LBL is concerned wholly with chromatophore control (Young, 1971), it is likely that each LBL cell has synaptic connections with more than one PCL cell.

The inhibition and excitation shown in PCL neurons on stimulation of the LBL tract could be caused in a number of ways (1) by inhibitory and excitatory LBL neurons that specifically inhibit or excite different PCL neurons, (2) from LBL neurons which have both excitatory and inhibitory synapses onto PCL neurons (Fig. 9.1.), or (3) through PCL interneurons. However, it was found that PCL neurons were either excitatory or inhibitory, but not both. Therefore, if a LBL cell has both excitatory and inhibitory synapses onto a PCL cell, one response would seem to dominate the other, during the LBL tract stimulation conditions used here. On the other hand if there are two types of LBL neurons, excitatory and inhibitory, then one would presume that stimulating the LBL would produce both expansion (excitation) and contraction (inhibition) of chromatophores on the skin. However, when the LBL of *Sepia* was stimulated (Boycott, 1961), the skin darkened and only expansion of the chromatophores occurred. It is more likely that each LBL neuron can make both excitatory and inhibitory contacts onto many PCL neurons. To achieve the fine control of expansion and contraction of chromatophores on the skin there would need to be a fine balance between the excitation and inhibition of PCL neurons. It is this balance that might control the frequency of tonic and other electrical activity in PCL motoneurons which could contribute to patterning on the skin.

LBL neurons themselves are smaller than PCL neurons and intracellular penetration was far more difficult. Consequently only a dozen LBL cell intracellular recordings were taken. However this is the first investigation to reveal the electrical activity of the LBL cells. It was found that LBL cells were either; 1) tonically active 2) showed bursting activity or 3) silent. EPSP activity was also revealed in tonically active cells when they were hyperpolarised. This synaptic activity was possibly present in many cells but may have been concealed by tonic activity and not revealed until the cell was hyperpolarised.

The morphology of the LBL cells lacked the complexity seen in PCL cells, which was a surprise, as one would expect that this higher centre of the brain would show more complexity. The cells were uniform, consisting of a long straight axon and a short simple array of dendrites with few arborizations. The differing patterns of dendrite branching seen between PCL and LBL cells must surely be significant. For instance, the long thin dendrites of some PCL cells presumably have different functions to the short stout ones of the LBL cells. Nevertheless this can only be speculated and in this study, more detailed work on the dendrite mechanics would need to be done. In common with the PCL cells, LBL cells showed swellings of various sizes and shapes on their dendrites and axons. The arborizations of the LBL cells were restricted to the neuropil presumably because the majority of synaptic contacts are made there. These results have opened up as many questions as they have answered.

Pharmacology.

It was found that the synaptic responses recorded from PCL cells could be blocked by a low calcium and high magnesium solutions. This provided evidence that the afferent evoked responses, seen in PCL cells, from the lateral basal lobe cells were mediated through chemical synapses. This is the first study to investigate the physiological effects of neurotransmitters at the cellular level in the PCL.

ACh behaved as an inhibitory neurotransmitter of tonic activity in PCL cells, and this is consistent with its paling effect on the skin when applied to the brain via the blood supply (Andrews et al, 1983). These results indicate that ACh would be the most likely neurotransmitter used at inhibitory synapses between LBL neurons and PCL neurons. Brain lesion experiments have implied that ACh acts at the level of the suboesophageal lobes to control chromatophores on the skin (Andrews et al, 1983). This view is supported by the results here reported and it is therefore likely that ACh receptors can be localised on PCL chromatophore motoneurons.

On application of 5-HT to the bath containing the brain slice, some PCL neurons showed an initial increase in the frequency of spontaneous activity with

the appearance of large EPSPs. This indicates that 5-HT may act as an excitatory neurotransmitter in the PCL. The 5-HT antagonist, methysergide, blocked the evoked synaptic component of an extracellular field potentials generated by LBL tract stimulation supporting the view that 5-HT has a role as an excitatory neurotransmitter in some LBL cells. 5-HT has a wide distribution in animals and singular 5-HT effects have been documented, for example the action of 5-HT includes acceleration of *Aplysia* heart (Leibeswar et al, 1975). These results, however, confirm for the first time Cornwall and Messenger's (1995) evidence that 5-HT is endogenous in the chromatophore motoneurons of *Alloteuthis.*

Some PCL neurons did not respond to 5-HT and consequently the role of 5-HT may not be the same for all PCL neurons. 5-HT might even have a dual purpose in the PCL or this may indicate the presence of two types of 5-HT receptors. A previous experiment performed on the octopus CNS, however, revealed that methysergide exclusively antagonized all 5-HT effects, indicating the presence of a single 5-HT receptor (Andrews et al 1983). These results have identified the presence of 5-HT receptors on PCL neurons. This confirms previous studies where it has been suggested that 5-HT receptors are located in the suboesophageal region of the brain (Andrews et al, 1983).

In octopus when 5-HT was applied to the brain via the circulation, it caused a mottled effect; expansion and contraction of chromatophores on the skin (Andrews et al, 1983). It was therefore expected that 5-HT would cause both excitatory and inhibitory effects in PCL neurons. But this was not found to be the case, possibly for several reasons (1) that *Octopus* is different from *Alloteuthis* (2) some PCL cells did not respond to 5-HT and perhaps this explains the mottling phenomenon (3) only a small sample of PCL neurons have been investigated so such conclusions should be made with caution, and (4) these mottling effects may originate from the optic lobe, which when stimulated produces recognised patterns on the skin (Boycott, 1961).

It has been shown in this study that the L-glutamate antagonists CNQX and GAMS blocked some evoked field potentials in PCL neurons. AP-4, on the other hand, had no effect at all, or slightly enhanced the evoked field.

The reduction in the synaptic responses produced by CNQX and GAMS indicates that in some cells L-glutamate acts as an excitatory neurotransmitter. L-glutamate is also the excitatory neurotransmitter at the chromatophore neuromuscular junction (Florey et al 1985). These results are also in agreement with the experiments of Andrews at al (1983) during which darkening on the skin occurred when L-glutamate was applied to the brain.

The enhancement of some evoked field potentials with the application of AP-4 requires another explanation, as here an additional source of current was revealed. This current may have been present before the application of AP-4 but may have been concealed, possibly due to summation with another field potential, possibly inhibitory, that was blocked by AP-4. Alternatively, a direct inhibitory input onto these cells, may have been blocked itself by the application of AP-4. Some cells showed no recognisable effects under the application of AP-4 or the other L-glutamate antagonists. However, AP-4 is not a highly specific antagonist of glutamate in mammals (Monaghan, 1989) so that its action in molluscs may well be unpredictable.

Overview of the chromatophore system.

Drawing together the previous morphological information (e.g. Dubas & Boyle 1985; Dubas et al 1986a&b; Novicki et al 1990) with that revealed here, and adding the new physiological data, we can begin to form an overview of how the squid posterior chromatophore system might operate, and how chromatophore patterns are organised by the CNS.

The vast majority of mantle body chromatophores are innervated by neurons with cell bodies in the PCL, although a small number of cell bodies may occur in the neighbouring fin lobe (Dubas et al, 1986a). From work on the octopus (Dubas & Boyle, 1985), it is likely that each motor axon innervates a number of chromatophores on different parts of the mantle and that a single motor unit is not coincident with a single chromatic unit. Thus, the chromatophore muscle fibres which are innervated by a single axon are involved in a particular pattern. This means that the repertoire of skin patterns is determined to a large extent in the periphery, by the morphological layout of the motor axon branches among the chromatophores. However, the number of chromatophores innervated by a single axon is quite small, varying between 2-60, with the large motor units situated on the fins where there is a high chromatophore density (Dubas et al, 1986a), and so a large number of motor units must be activated synchronously to produce all but the simplest patterns.

There are a number of ways to select and activate a population of motoneurons within the PCL to produce individual patterns. One way would be to group all those neurons involved in a particular pattern close together and activate them by a single or small group of neurons from higher centre (e.g. the LBL). Now, although there is very little sign of a somatotopic correlation between the position of the chromatophore on the mantle and the position of the motoneuron cell body in the PCL (Dubas et al, 1986a), this does not exclude this model, for most of the connections will be through the dendrites and not directly on the cell bodies. From this then we might conclude that the more complex patterns, where large numbers of motor units are recruited, might involve cells with large dendritic trees, i.e. cell type II, and possible III, as described in this

thesis. Of course, not all synapses on a dendritic tree need be activated at once, and it may be that the large dendritic trees also indicate the flexibility of the cell, in that it can be brought into a number of different patterns, by recruitment through different synaptic paths.

Two other novel mechanisms have been revealed in this work that will most likely contribute to the formation of patterned outputs. The first is electrical coupling between motoneurons. Clearly, it is relatively simple to build a chromatophore pattern by coupling together the motoneurons so that they fire together. A disadvantage of this system is that it would be very inflexible if there was no way of modifying the coupling, so that different cells could be coupled and uncoupled depending on the pattern needed. However, Williamson & Chrachri (1994) have shown that sensory hair cells in the squid statocyst are electrically coupled but that this coupling can be modulated by the activity of efferent neurons from the brain. It is to be expected therefore, that the level of electrical coupling between PCL neurons could also be modulated and thus form different patterns of output from the coupled cells. The second feature seen in these experiments was the presence of interneurons. Young (1976) doubted whether interneurons were present in the squid PCL, but the dye fills from this study clearly show small cells whose processes are entirely contained within the PCL. Unless this is some quirk and the cells are only partially filled, these must be identified as local interneurons. Such interneurons have also identified in the octopus PCL (Miyan & Messenger, 1995) and it is to be expected that these will play a major role in orchestrating the output from the PCL either by directly connecting motoneurons within the PCL or by acting as intermediary cells between incoming neurons from, for example, the LBL and PCL motoneuron.

The main input to the PCL has been shown to be from the ipsilateral LBL, although there are some tracts from other areas, such as from the anterior pedal lobes (Novicki et al, 1990). The LBL to PCL tract spread thoughout the neuropil area of the PCL and are therefore well placed to exert a major influence on the activity of these PCL neurons. LBL cells have been shown in this study to cause both inhibition and excitation of different PCL neurons and it seems likely that each LBL neuron could depress or excite the ongoing activity in individual PCL

motoneurons and interneurons, through pathways as illustrated in Fig.9.1. Thus, although individual chromatic units are likely to rely on the activation of single or small numbers of motoneurons, these can be linked together within the PCL by coupling (electrical or chemical), perhaps using interneurons, or directly through dendritic contact, to form larger patterns, which are then modified or orchestrated by inputs from the LBL to form whole displays. This fine control from the LBL will use both excitation and inhibition of ongoing activity to recruit or detach different groups of motoneurons and interneurons from the pattern. The inhibition seems to be associated with acetylcholine as the neurotransmitter and the excitation with 5-HT and perhaps glutamate.

This is obviously not the full story for the details of how this organised at the individual cell level, and the interconnections that permit it, are still missing. But perhaps, now knowing the broad brushstrokes, and having the physiological methods of investigation available, it will be possible to build on the excellent morphology data, and derive the detailed circuits involved in the production and control of chromatophore displays.

9.3 Summary and future work.

The major contribution of this work has been to demonstrate that a brain slice preparation can be successfully used to study nervous activity and neural connections in cephalopod brains. This is a great step forward, for although there are numerous detailed descriptions of cephalopod behaviours and the amazing learning capabilities of these animals, there has been almost no work done on the neurophysiological basis of such capabilities, and this is despite the excellent information available on the anatomy of their nervous systems. In addition, this work has contributed to our understanding of the squid chromatophore system by showing that (1) there are at least four main morphological categories of cell types within the Posterior Chromatophore Lobe (PCL) and that these can be correlated with specific electrical characteristics, (2) electrical coupling exists between PCL neurons, possible contributing to the formation of patterned outputs from the system, (3) the neurotransmitters ACh, 5-HT, and glutamate are employed in the PCL system, and (4) stimulation of the Lateral Basal Lobe (LBL) to PCL tract has direct effects on PCL neurons and these can be inhibitory or excitatory.

It is clear that the results presented in this work have gone some way towards answering the difficult questions surrounding the neural control of cephalopod colour change outlined in the Introduction. However, it is apparent that this is only the first small step towards understanding a neural system that rivals in complexity many of those found in higher vertebrates. There are obvious ways of further using and developing this preparation, both at the systems level and the level of individual neurons. For example, the pharmacology and ion channel characteristics of individual cells can now be examined, or the connections between PCL chromatophore motoneurons can be studied, looking at how much they act in concert to produce patterns of chromatophore activity, whether this is through connections between motoneurons, through PCL interneurons, or if it is all organised from the LBL or above.

As with all neurophysiological studies of brain function, there is never an end, but only new questions to be answered based on the findings already outlined. The cephalopod chromatophore system may not attract the hundreds of

researchers now studying for example, the vertebrate visual system, by it nevertheless has the potential to reveal how a complex motor output can be generated and controlled and perhaps give some clues to the fundamental mechanisms at work in such sophisticated systems.

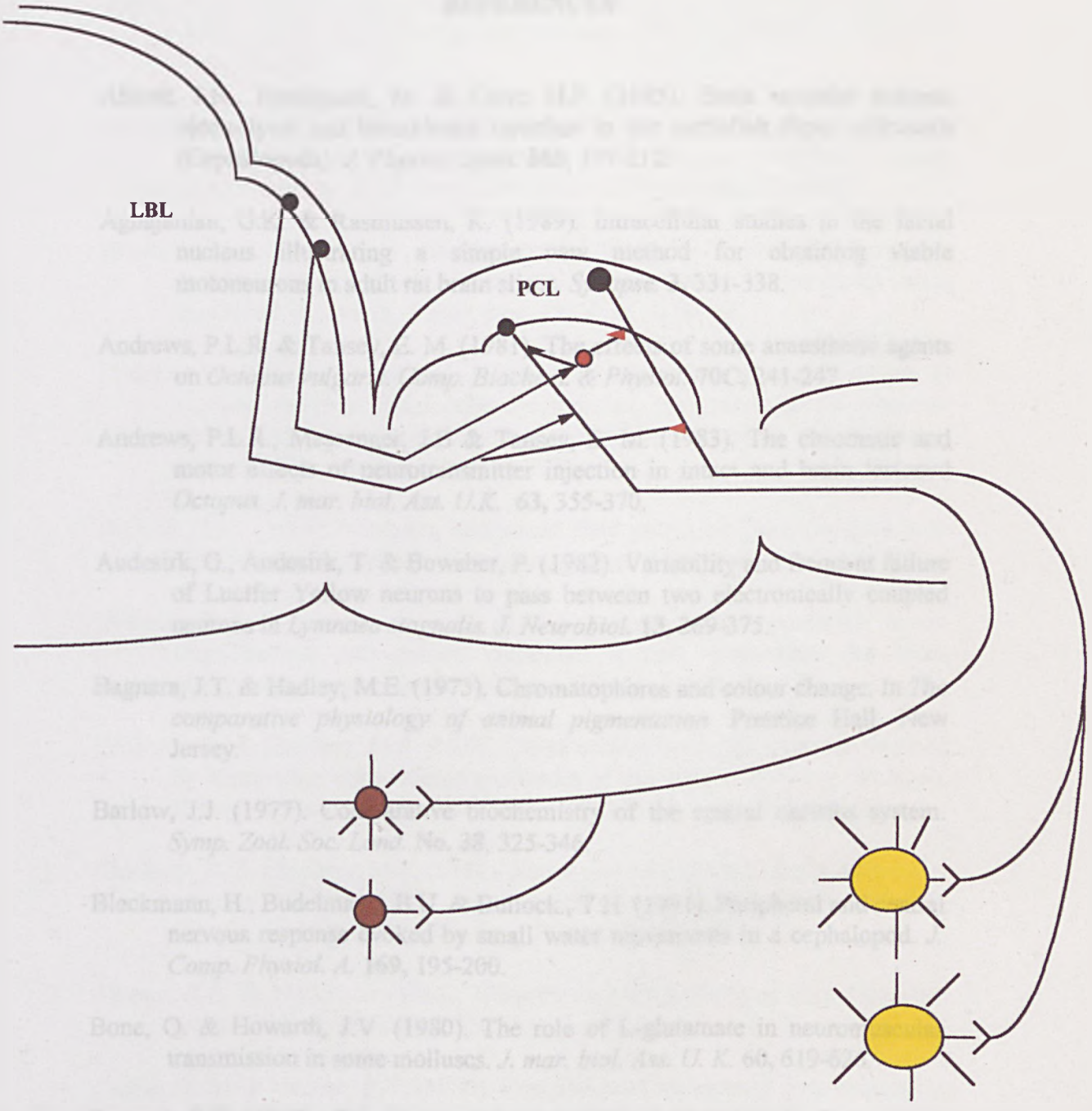


Fig 9.1 Summary diagram of the input pathway from the LBL and how this input is likely to be involved in the chromatophore control of *Alloteuthis subulata*. The brain is shown in sagittal section, with the pathway shown only on one side. Circles represent the origin of the pathway (somata) and the arrowheads represent inhibitory (Black) and excitatory (Red) synapse. Each LBL cells can be seen making both excitatory and inhibitory contacts onto multiple PCL chromatophore motoneurons, either directly or indirectly via PCL interneurons (Red circle). The LBL axons are shown to synapse with the PCL axons in the PCL neuropil as field potential data indicates this to be the case. The effect of either the inhibitory or excitatory influence of the LBL cells can be seen on the skin, with contraction (darkening) of the chromatophore with inhibition of the PCL motoneuron, and expansion (lightening) with excitation. For simplicity only two LBL and PCL neurons have been shown.

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