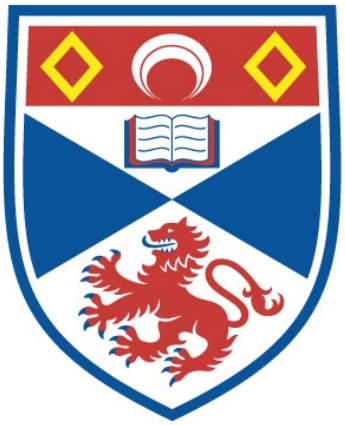
A STUDY OF THE ACTION, AND THE MECHANISM OF ACTION OF SEROTONIN ON PYRAMIDAL NEURONES IN THE HIPPOCAMPAL SLICE PREPARATION

Peter J. R. Cobbett

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the
University of St Andrews
for the degree of Doctor of Philosophy.

by

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SUMMARY

1) Transverse hippocampal slices were prepared from rats and maintained in a recording chamber. Intracellular recording techniques were used to measure the amplitudes of the resting potentials (mean -63.4mV), the action potentials (mean 72.5mV), and the input resistance (mean 16.5MΩ) of neurones in the CA1 region of the hippocampus. Impalements of neurones could be maintained for up to 9.5hrs.

2) Impaled neurones in the CA1 region of the hippocampus were identified as pyramidal neurones after injection of the fluorescent dye Lucifer Yellow CH and subsequent microscopical examination of the slices. The projections of the apical and basal dendrites were complete and in some cases the axon could be identified and traced toward the subiculum. Injections of pyramidal neurones in the CA3 region and of granule cells in the area dentata demonstrated the different morphologies of the three types of neurone.

3) CA1 neurones were hyperpolarised by up to 10mV by serotonin applied locally by iontophoresis. This response was slow on onset and lasted between 30s and 3min. In contrast, glutamic acid and acetylcholine depolarised these neurones.

4) The amplitude of the hyperpolarisation induced by serotonin was dependent on the amplitude and duration of the iontophoresis current. A decreased input resistance of the neurones was associated with and followed the same time course as the potential

change induced by serotonin.

5) The serotonin response was reversed at membrane potentials more negative than the reversal potential which was between -81mV and -104mV. This high reversal potential indicated that the response might be mediated by efflux of potassium ions from the neurones.

6) In ion substitution experiments, the serotonin response was shown to be dependent on the extracellular potassium ion concentration, but was independent of the chloride ion concentration.

7) The serotonin response was blocked by the putative serotonergic antagonist methysergide (100μM). However, the responses of the neurones to serotonin were not affected by cyproheptadine, methergoline, mianserin, and l-propranolol which have also been proposed to be antagonists of serotonin in the central nervous system.

8) The significance of these results is discussed with reference to previous investigations by other workers.

ACKNOWLEDGEMENTS

I would like to thank Dr Glen Cottrell, my supervisor, for his encouragement and support during the period in which the research was undertaken. I should also like to thank Dr D.N. Middlemiss, I.C.I. Ltd., for his comments as the research progressed.

I would like to take this opportunity to thank Mr C. Rommele and Mr R. Jack of the Gatty Marine Laboratory, and Mr B.Powell for their excellent technical assistance. I also thank I.C.I. Ltd. for financial assistance and Dr W. Stewart for the kind donation of Lucifer Yellow CH.

Finally my thanks go to my parents for my education, and to my wife, Sue, for her support and understanding.

ACADEMIC RECORD

I first matriculated at the University of St Andrews in October 1972 and graduated with the degree of B.Sc. (Hons) in Physiology in 1977.

I matriculated as a postgraduate student of the Department of Physiology & Pharmacology, University of St Andrews, in October 1977.

CERTIFICATE

I hereby certify that P.J.R.Cobbett has spent twelve terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court, No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Declaration

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of St Andrews entitled "A study of the action, and mechanism of action of serotonin on pyramidal neurones in the hippocampal slice preparation" is my own composition and is the result of work done by me during the period of matriculation for the above degree. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology & Pharmacology, United College of St Salvator and St Leonard, University of St Andrews, under the supervision of Dr Glen Cottrell.

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 SEROTONIN IN THE MAMMALIAN CNS

Serotonin (5-hydroxytryptamine) is one of a number of substances that have been proposed as transmitter candidates in the mammalian central nervous system (CNS). Serotonin was first isolated in the brain by Twarog & Page (1953), and was shown to be unevenly distributed in the brain shortly afterwards (Amin, Crawford & Gaddum, 1954). Confinement of serotonin to specific neurones was demonstrated biochemically (Heller, Harvey & Moore, 1962), and histochemically (Dahlstrom & Fuxe, 1965). The perikarya of the serotonin-containing neurones were isolated in the raphe nuclei of the midbrain: the median (B8) and the dorsal (B7) raphe nuclei being the largest source of serotonergic neurones projecting to forebrain areas. Lesions of the raphe nuclei decreased levels of serotonin and enzymes for its synthesis in the brain, but other transmitter systems were not affected (Lorens & Guldberg, 1974; Jacobs, Wise & Taylor, 1974). Many brain nuclei have been shown to receive a differential serotonergic innervation from the raphe nuclei: lesions of one raphe nucleus produced a greater decrease of the serotonin concentration in these nuclei than lesions of the other nucleus (Jacobs, Wise, & Taylor, 1974; Van de Kaar, & Lorens, 1979).

Recently a denser and more extensive distribution of serotonin containing terminals in the brain has been shown. This distribution was demonstrated with the techniques of autoradiography (Conrad, Leonard, & Pfaff, 1974; Moore & Halaris, 1975; Azmitia & Segal, 1978), and anti-body labelling (Lidov,

Grzanna & Molliver, 1980). The density of the total and differential innervations of a brain nucleus by the raphe nuclei demonstrated by these two techniques correlate with the total and differential contributions to the serotonin concentration of that nucleus.

Although the roles of any single transmitter candidate in the CNS are not clear, central transmitter systems are important in the control of mood and of general activity states. Impairment of a specific transmitter system may be characterised by stereotypic abnormal behaviour. Changes of the activity of a transmitter system may be induced or reversed by drugs acting presynaptically or postsynaptically. Postsynaptically, endogenous and applied substances interact with receptors to inhibit or mimic the effects of the transmitter.

The central interactions of serotonin with postsynaptic receptors have been studied at the subcellular level in vitro, and at the cellular level in vivo, and at the whole animal level. These studies, from which models for the interaction of serotonin and its receptors have been devised, have often been concerned with the effects of putative serotonergic antagonists. As yet, potent and specific central serotonin antagonists, which might improve our understanding of the central roles of serotonin are not available. In the rest of this chapter, I will outline some of the evidence that serotonin interacts with receptors on the postsynaptic membrane and how these interactions have been studied. Particular reference will be made to five putative antagonists:

cyproheptadine, methergoline, methysergide, mianserin, and l-propranolol.

Serotonin has also been isolated in mammalian peripheral tissues, and/or has been found to be active in the periphery. Peripheral transmitter-receptor interactions have been studied to determine the similarity of central and peripheral serotonergic receptors. Interactions between serotonin and its receptors have also been studied in invertebrates where there is more complete evidence for serotonin as a transmitter at identified synapses. Peripheral mammalian preparations and preparations from invertebrates have also been used as models for investigation of the effects of serotonin

1.2 INTERACTIONS OF SEROTONIN WITH POSTSYNAPTIC RECEPTORS

1.2A Biochemical Studies.

The regional distribution and types of central postsynaptic serotonin receptors have been studied biochemically using *in vitro* radio-ligand binding techniques. In these studies, the amount of specific binding of tritiated serotonin, a serotonin analogue, or a serotonergic antagonist to synaptic membranes was measured in the absence and presence of binding inhibitors. These studies have shown that : 1) There are at least two serotonin receptor types in the brain; one type has a high affinity for serotonin and the second has a high affinity for spiroperidol, and both types have equal affinity for d-LSD (Bennett & Snyder, 1976; Peroutka &

Snyder, 1979). 2) Receptor binding is inhibited by serotonin analogues such as 5-methoxy N,N, dimethyltryptamine (5-MeODMT) (Bennett & Snyder ,1976). 3) Receptor binding is inhibited by methysergide, cyproheptadine, and by d-LSD, but not l-LSD, (Bennett & Snyder, 1975), by mianserin (Peroutka & Snyder, 1979), by methergoline (Fuxe, Ogren, Agnati & Jonsson, 1978), and by l-propranolol, but not by d-propranolol, (Middlemiss, Blakeborough & Leather, 1977). 4) Some neuroleptics were more potent as inhibitors of serotonin binding to serotonergic receptors, than as inhibitors of dopamine binding to dopaminergic receptors (Peroutka & Snyder, 1981).

1.2B Electrophysiological Studies.

In cats, guinea pigs, and rats, the frequencies of spontaneous, glutamate induced, and electrically induced firing of most neurones in vivo were reduced by locally applied serotonin, but some neurones were excited (see Bloom, Hoffer, Siggins, Barker & Nicoll, 1972, for a review). However some of the excitatory responses may have been produced by application of serotonin at low pH, so that the excitation was produced by H^+ rather than serotonin (Jordan, Frederickson, Phillis & Lake, 1972). It has also been pointed out that: 1) in those areas receiving a prominent innervation from the raphe nuclei, serotonin was usually inhibitory, and 2) serotonin was usually excitatory in those areas with a sparse innervation from the raphe nuclei (Haigler & Aghajanian, 1974, 1977; Aghajanian & Wang, 1978). These workers also showed that methergoline and cyproheptadine were only

effective as central serotonergic antagonists where serotonin was excitatory, but methysergide blocked both excitatory and inhibitory responses. Similar observations were made by Segal (1975, 1976) who showed that serotonin reduced the firing rate of hippocampal neurones of rats in vivo. This serotonin response was blocked by methysergide, partially blocked by cyproheptadine, and not affected by mianserin. The only reported effects of propranolol showed that d,l-propranolol, but not d-propranolol, reduced the serotonergic inhibition of cerebellar purkinje cells in vivo (Yeroukalis, Alevizow, Gatzonis & Stefanis, 1981).

Recently, serotonin and noradrenaline have been shown to facilitate the excitatory effects of glutamate on neurones in the facial motor nucleus (VanderMaelen & Aghajanian, 1980; McCall & Aghajanian, 1981). Facilitation of the response by serotonin, but not by noradrenaline, was blocked by methysergide, methergoline and cyproheptadine. d,l-Propranolol had no effects on either serotonin or noradrenaline facilitation of the glutamate response.

1.2C Behavioural Studies.

Two models of animal behaviour have been used to represent increased central serotonergic receptor stimulation. First, administration of either of the serotonin precursors l-tryptophan or 5-hydroxytryptophan (5HTP), together with a monoamine oxidase inhibitor, increased levels of serotonin in the brain, and "spillover" of serotonin from the presynaptic neurones. The consequent overstimulation of postsynaptic serotonin receptors

produced stereotypic behaviour including shaking or weaving of the head (Corne, Pickering & Warner,1963). This behaviour was also produced by the serotonin analogue 5-methoxy N,N dimethyltryptamine which is a mimetic of serotonin (Green & Grahame-Smith, 1978). The behavioural effects of l-tryptophan, 5-HTP and 5-MeODMT were partially or completely blocked by cyproheptadine (Corne, Pickering & Warner, 1963; Matthews & Smith, 1980; Weinstock & Weiss, 1980), by methergoline (Ferrini & Glasser, 1965; Fuxe, Ogren, Agnati & Jonsson, 1978; Matthews & Smith, 1980; Deakin & Green, 1978), by methysergide (Corne, Pickering & Warner, 1963; Matthews & Smith, 1980; Sloviter, Drust, Damiano & Connor, 1980), by mianserin (Sloviter, Drust, Damiano & Connor, 1980), and by l-propranolol, but not d-propranolol, (Green & Grahame-Smith, 1976; Costain & Green, 1978; Weinstock & Weiss, 1980).

The second model for increased receptor activation is serotonin induced sleep in the chick (Spooner & Winters, 1965). Mianserin and methysergide inhibited serotonin induced sleep completely, but cyproheptadine was less potent and l-propranolol was ineffective (Jones,1980). However, in an earlier study, l-propranolol, but not d-propranolol, effectively blocked serotonin induced sleep (Weinstock, Gitter & Weiss, 1973).

1.2D Studies on Peripheral Mammalian and Invertebrate Serotonin Receptors.

Several models of serotonin action in mammalian peripheral tissues have been used to test the effects of putative antagonists.

Serotonin produces contraction of the rat uterus *in vitro* and of the stomach fundic strip, and produces bronchoconstriction in guinea pigs. Cyproheptadine, methysergide, methergoline, mianserin, and l-propranolol but not d-propranolol have been shown to be active as serotonin antagonists in at least one of these tissues (Stone, Wenger, Ludden, Stavorski & Ross, 1961; Beretta, Ferrini & Glasser, 1965; Vargaftig, Coignet, de Vos, Grijsen & Bonta, 1971; Schechter & Weinstock, 1974).

In gastropod molluscs, there is the best evidence for serotonin as a neurotransmitter in invertebrates. In the CNS there is an identified serotonergic system (Cottrell, 1970;Cottrell & Macon, 1974). Central neurones have been shown to have five different types of response to locally applied serotonin (Gerschenfeld & Paupardin Tritsch, 1974). However methysergide (Cottrell & Macon, 1974) and d- or l- propranolol (Cottrell & Cobbett, 1978; see also Appendix) have been shown to be only weak antagonists of serotonin.

1.2E Serotonin as a Central Neurotransmitter.

There is good, if sometimes indirect, evidence that serotonin is a neurotransmitter in the mammalian CNS, but the mechanisms of serotonin action are not clear. Electrophysiological studies *in vivo* have shown that serotonin alters the firing rate of many neurones, but the underlying effects on the membrane are not known. At the subcellular level, investigations have shown which substances interact with serotonergic receptors but can not show

the activity of these substances as agonists or antagonists. Conversely studies on serotonin and its putative antagonists at the whole animal level are subject to changes in other transmitter systems as well as the serotonergic systems. The activities of the five putative antagonists from these electrophysiological, biochemical and behavioural studies are not clear: in some circumstances, the five compounds appear to be potent serotonergic antagonists but in other cases to have no effect.

It therefore seemed appropriate to investigate more closely the effects of serotonin on central neurones, and the effects of these putative antagonists. To examine the effects of serotonin on neuronal membranes which underlie changes of firing rate, intracellular recording techniques must be used. Although in vivo recordings may be made, the techniques are difficult, and the results may be complicated by the effects of anaesthetics. Two preparations have been developed which have advantages over the in vivo experimental situation: first, brain slices prepared usually from rats, guinea pigs or rabbits and second, cultured neurones produced from the spinal cord or the brain of foetal mice. Both offer the advantages of a mechanically stable preparation, in which electrodes can be positioned under visual control, and in which the extracellular environment may be altered. The major advantage of slices over cultured neurones is that the structural relationship of neurones is more completely maintained in slices.

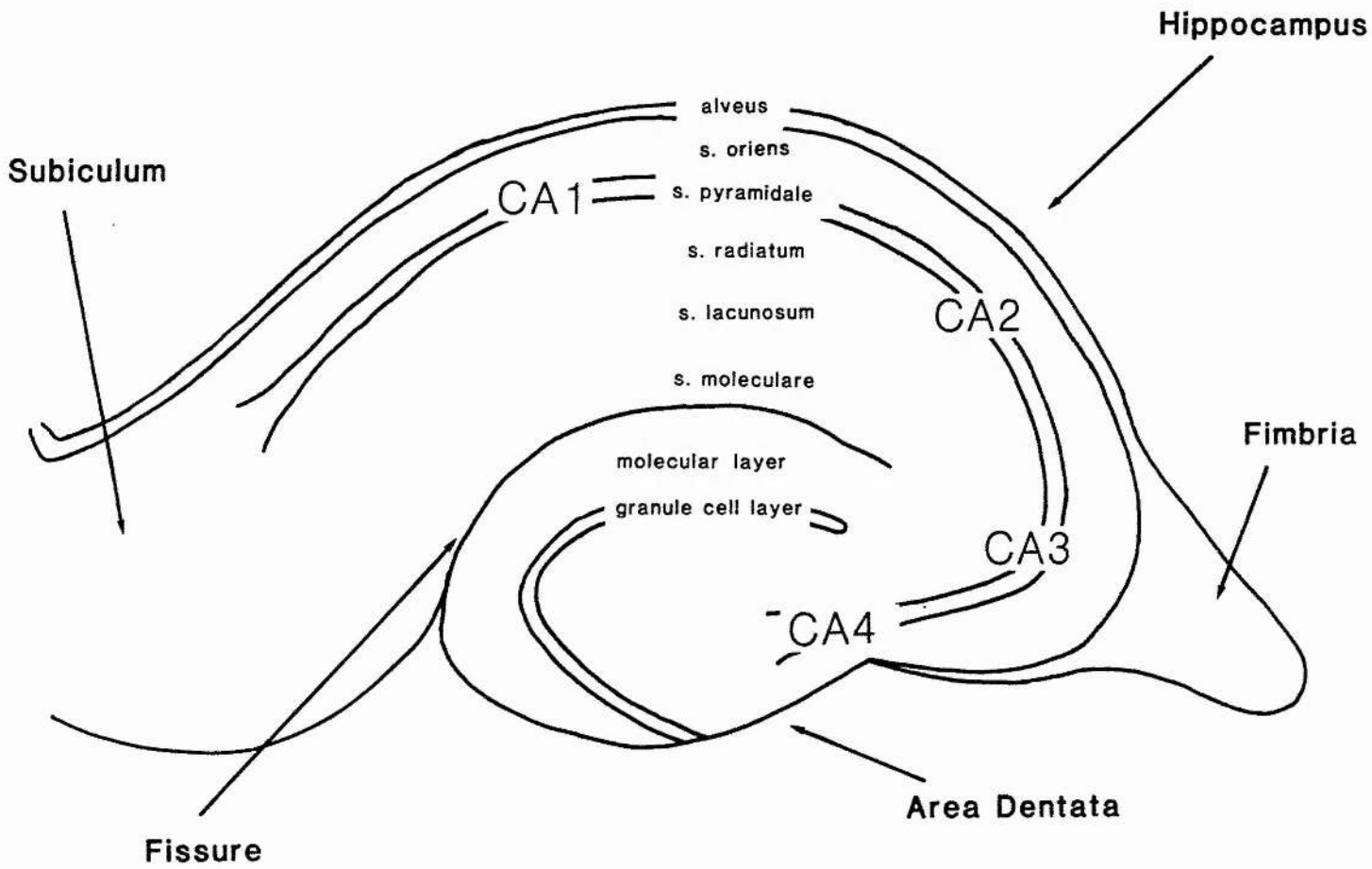
1.3 A BRAIN SLICE PREPARATION FOR THE STUDY OF THE ACTIONS OF SEROTONIN

Brain slices have been used in biochemical and physiological studies since Chuh Loh & McIlwain (1957) showed that resting potentials could be recorded from neurones in slices of the cerebral cortex. Studies have also been made using slices prepared from other brain nuclei as well as the cortex (Dingledine, Dodd & Kelly, 1980). Normal synaptic phenomena and the effects of changes of the extracellular medium on these phenomena have been investigated using extracellular and intracellular recording techniques. Where intracellular recording techniques have been used, normal resting potentials have been recorded and these may be constant over several hours indicating that slices were in good metabolic condition. In addition, the effects of drugs on evoked synaptic activity, on the resting potentials, and on spontaneous and evoked firing can be studied.

To investigate the central actions and mechanisms of action of serotonin, slices should be prepared from a brain area in which there is good evidence that serotonin acts as a neurotransmitter. One region of the brain which receives a serotonergic input and which, in addition, is structurally "simple" is the hippocampal formation.

The structure of the hippocampal formation of rodents has been extensively investigated (Cajal, 1911; Lorente de No, 1934). The formation is divided into two regions: the hippocampus and the

Fig. 1.1 Drawing of the hippocampal formation of the rat in transverse section, showing its anatomical divisions. The formation is divided into two major parts: the hippocampus, which is divided into four regions (CA1 - CA4) and into six layers or strata (s) including the alveus, and the area dentata, which is divided into two layers. The hippocampus and area dentata are separated by a fissure, and their extrinsic connections are made through the fimbria and the subiculum.


Hippocampus
alveus
s. oriens
Subiculum
CA1
s. pyramidale
s. radiatum
s. lacunosum
CA2
s. moleculare
Fimbria
molecular layer
granule cell layer
CA3
CA4
Area Dentata
Fissure

area dentata (See Fig. 1.1). The hippocampus has been divided into four regions (CA1-CA4) based on the structure of the pyramidal neurones and their axonal projections. It has also been divided into six layers or strata including the alveus which are distinguished by the structure of the pyramidal neurones and their afferent connections in each layer. The area dentata is divided into two layers, and the granule cells of the area dentata are a homogeneous population with the same efferent and afferent projections.

The hippocampus in the rat contains serotonin (Saavedra, Brownstein & Palkovits, 1974) and receives a serotonergic input from the raphe nuclei (Conrad, Leonard, Pfaff,1974; Moore & Halaris,1975). The serotonergic input is mostly from the median raphe nucleus, but there is a contribution from the dorsal raphe (Jacobs, Wise & Taylor, 1974; Azmitia & Segal, 1978). In the CA1 region, the serotonergic innervation is most dense in the stratum lacunosum and the stratum moleculare (Moore & Halaris, 1975;. Lidov, Grzanna & Molliver, 1980). The anatomical identification of a serotonergic innervation has been confirmed physiologically. Stimulation of the raphe nuclei reduces the firing rate of the CA1 neurones and this effect is mimicked by local application of serotonin (Segal & Bloom, 1974; Segal, 1975).

The electrophysiology of hippocampal neurones in vivo has been extensively investigated in cats (Kandel, Spencer & Brinley, 1961; Kandel & Spencer, 1961; Spencer & Kandel, 1961,a; Spencer & Kandel, 1961,b). In guinea pig hippocampal slices, which were

first shown to be viable by Skrede & Westgaard (1971), the properties of the CA1 pyramidal neurones have been found to be similar to those of CA3 pyramidal neurones in cats *in vivo* (Schwartzkroin, 1975, 1977). Synaptic phenomena previously described in the hippocampus *in vivo* have also been show to occur in hippocampal slices.

1.4 AIMS OF THE STUDY

There were three major aims of this study:-

1) To record the effects of serotonin on the CA1 pyramidal neurones in hippocampal slices.

2) To examine the ionic basis of the serotonin response(s) of the pyramidal neurones.

3) To examine the effects of cyproheptadine, methergoline, methysergide, mianserin and l-propranolol as antagonists to serotonin in hippocampal slices.

To achieve these aims, it was first necessary: 1) To produce slices from the hippocampus of rats and maintain them in a recording chamber. 2) To make satisfactory penetrations of CA1 neurones and to identify the neurones. 3) To show that the extracellular fluid of the slices was in equilibrium with the artificial cerebro-spinal fluid (ACSF) in the recording chamber.

CHAPTER TWO

METHODS

2.1 INTRODUCTION

The techniques for preparing and maintaining brain slices for electrophysiological studies have evolved from those used by Chuh-Loh and McIlwain (1957). It is still necessary to follow the same basic protocol. Slices must be prepared rapidly after sacrificing the animal, and must be maintained at a suitable temperature, well oxygenated, and bathed by an artificial cerebro-spinal fluid, ACSF.

Hippocampal slices, prepared from guinea pig or rat brains, have been cut in three ways: 1) with a hand held razor blade (Skrede & Westgaard, 1971), 2) with a tissue chopper in which the blade is moved rapidly, vertically, through the tissue (Schwartzkroin, 1975), and 3) with a Vibratome in which the blade vibrates as it moves slowly through the tissue (Dingledine, Dodd & Kelly, 1977). Each of these methods has certain advantages. For instance, when a Vibratome is used, tissue is cut while completely immersed in warmed and well oxygenated ACSF. On the other hand, the whole hippocampus may be sliced transverse to the longitudinal axis with a tissue chopper, but not with a Vibratome.

The recording chambers used in slice studies by other workers have have been of two basic designs. In the first type, the slices were positioned on a nylon net with only the lower surface of each slice in direct contact with ACSF (Skrede & Westgaard, 1971; Haas, Schaerer & Vomansky, 1979). In the second type of chamber, the slices were completely immersed in ACSF and were held between two

ION	CSF	ACSF
Na^+	149	150
K^+	2.9	6.25
Mg^{++}	1.74	2.0
Ca^{++}	2.47	2.0
Cl^-	130	133
HCO_3^-	22	26
Glucose	5.35	10
pH	7.37	7.35

Table 2.1 Comparison of the concentrations of ions and glucose, and the pH of CSF (Davson, 1967) and ACSF (Schwartzkroin, 1975). The concentrations are given as mequiv/kg H_2O for CSF, and mM for ACSF.

nylon nets (Schofield, 1978). With the first type of chamber, electrodes are more easily positioned on slices but the upper surfaces of the slices are liable to become dry.

The ACSF bathing the slices in the recording chamber should have an ionic composition similar to that of cerebro-spinal fluid, CSF. However the ACSF used by most workers has differed from the composition of CSF in two respects. First, based upon the concentration used in early biochemical studies on brain slices, the potassium concentration in ACSF was higher than that in CSF. Second, to increase the stability of intracellular recordings, the calcium concentration in ACSF was higher than that in CSF. The ionic composition of ACSF (Schwartzkroin, 1975) and of CSF (Davson, 1967) are compared in Table 2.1.

The intracellular recording techniques which have been used in slice studies are similar to those used to record from other neurones in vitro. Most workers have used voltage recording techniques, but some recent investigations have been made using single electrode voltage clamp techniques (Johnston & Hablitz, 1979; Adams, Brown & Halliwell, 1981; Johnston, 1981).

The methods described below for preparing and maintaining slices, and for recording from neurones in the slices are based on those described by others. However, some modifications have been made for particular experiments and in attempts to improve the quality of recordings.

2.2 ARTIFICIAL CEREBRO-SPINAL FLUID

2.2A Ionic Composition.

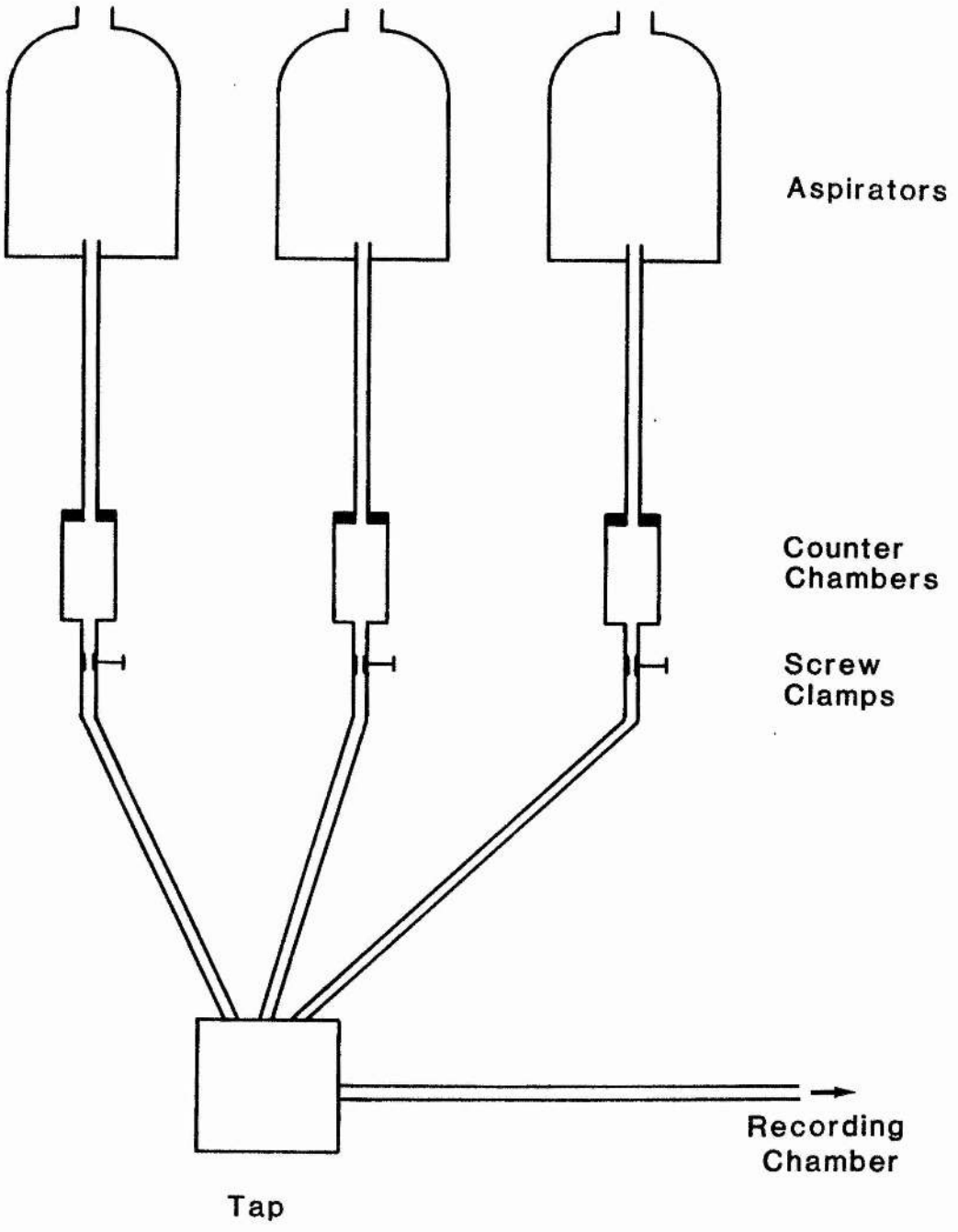
In the first experiments, the ionic composition of ACSF was the same as that used by Skrede & Westgaard (1971) and by many other workers. ACSF was made up from concentrated salt solutions which were diluted to the final volume with fresh distilled water. To this final volume glucose was added as required. The concentrated salt solutions were kept for up to 14 days at 4°C and then discarded. These solutions were made up with fresh distilled water and were filtered before storage (Whatman Number 1, qualitative filters). These precautions were taken after small algal and fungal growths were observed in unfiltered concentrated solutions kept for longer periods or at room temperature. As a further precaution, ACSF was only used on the day it was made. Table 2.2 shows the composition of the concentrated solutions and the concentration of each in the normal ACSF.

In later experiments, two changes were made to the composition of normal ACSF. The K^+ concentration was reduced to 3.75mM from 6.25mM by reducing the amount of KCl in ACSF (see Table 2.2). The Ca^{++} concentration was increased from 2mM to 4mM by addition of $CaCl_2$ after the "incubation period" (see below). Except where stated, the data presented in Chapters 3, 4, and 5 have been obtained from experiments in which the bathed in ACSF with 3.75mM K^+ and 4mM Ca^{++} concentrations.

SALT	STOCK SOL'N	$ACSF_1$	$ACSF_2$
	M	mM	mM
NaCl	2.48	124	124
KCl	0.5	5	2.5
KH_2PO_4	0.25	1.25	1.25
$NaHCO_3$	0.52	26	26
$MgSO_4$	0.4	2.0	2.0
$CaCl_2$	0.4	2.0	2.0 (4.0)
Glucose		10	10

Table 2.2 The composition of the concentrated salt solutions, and the final concentration of each solution in ACSF.
$ACSF_1$ was the normal ACSF, used in the first experiments, in which the K^+ concentration was 6.25mM and the Ca^{++} was 2mM throughout the experiments.
$ACSF_1$ was the normal ACSF, used in most experiments, in which the K^+ concentration was 3.75mM and the Ca^{++} concentration was raised from 2mM to 4mM at the start of the recording phase in each experiment.

Fig. 2.1 Diagram showing the arrangement of the apparatus to introduce ACSF from one of three aspirators into the recording chamber, and to monitor and control the rate of flow of ACSF from each aspirator. Up to three ACSF solutions could run through the tap simultaneously. From the tap, one solution flowed to the recording chamber and the other solutions flowed to waste (not shown).

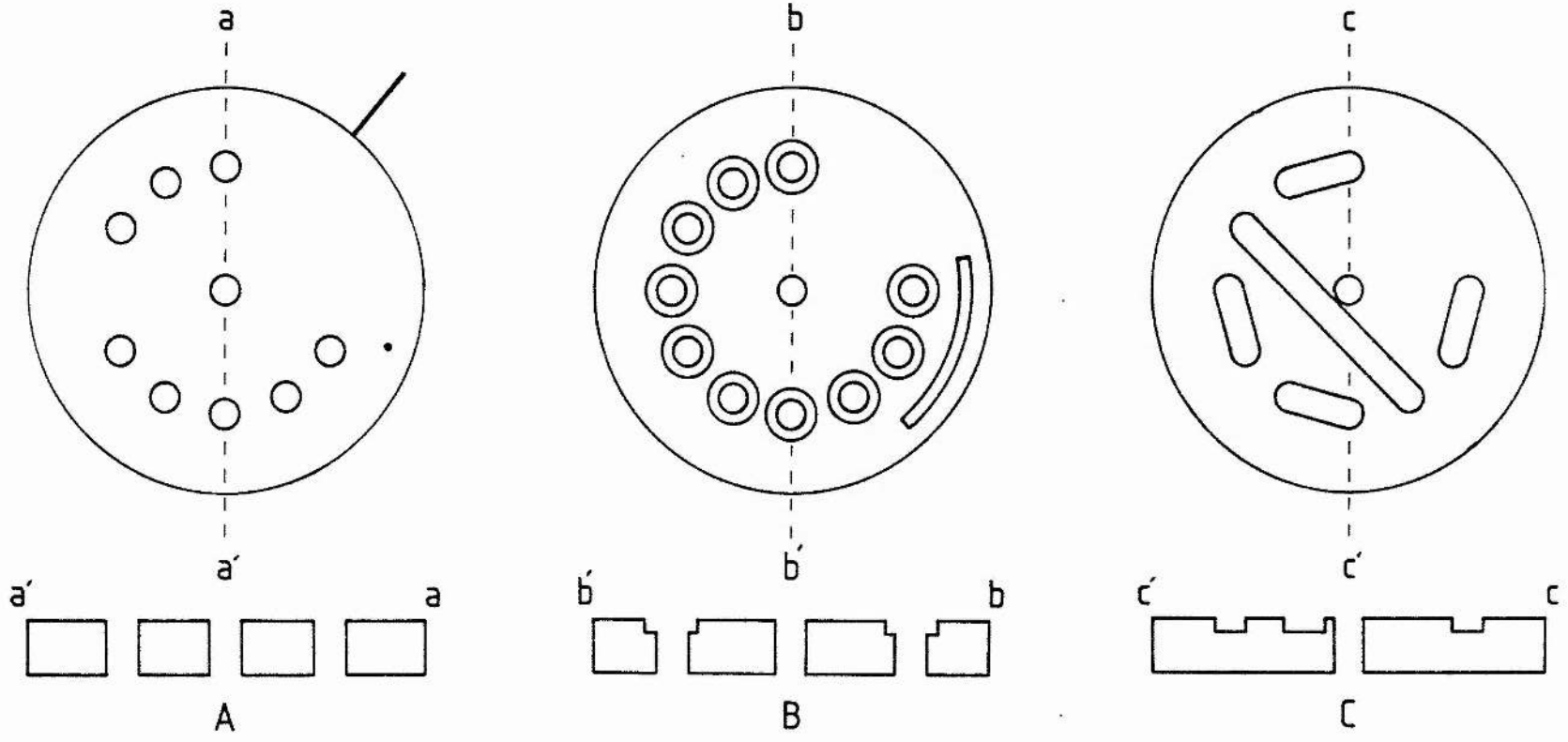

Aspirators
Counter Chambers
Screw Clamps
Recording Chamber
Tap

In some experiments the ionic composition of the ACSF was changed during an impalement. These changes were made by adding to, or omitting from, the ACSF solution the correct amount of the relevant concentrated salt solution. When such changes of composition were considered to produce an excessive osmolarity change, the ionic composition was altered by substitution of one ionic species with another. Details of these changes are given in the relevant results sections.

2.2B Introduction to the Recording chamber.

ACSF solution was introduced into the recording chamber from three aspirator bottles about 0.5m above the level of the recording chamber (Fig. 2.1). The solution in each aspirator was bubbled with a mixture of oxygen and carbon dioxide (95%:5%) to oxygenate the ACSF and to bring the pH of the ACSF to 7.35. ACSF flowed from each aspirator in polythene tubing via a simple drip counter. The rate of flow was controlled by a screw clip on the tubing leading from each counter chamber. At the level of the recording chamber, the three polythene tubes were lead into a tap mechanism so that the source of ACSF entering the recording chamber could be controlled. Initially a twelve way tap (Partridge & Thomas, 1975) was used. However, with this tap, small changes of flow rate of ACSF into the recording chamber occurred during and following changes between sources of ACSF. Such changes always resulted in the loss of the impalement. Subsequently, a three way tap was developed so that impalements could be maintained when the source of ACSF was changed.

Fig. 2.2 Drawings of the three discs which form the mechanism of the three way tap. Drawings A, B, & C, refer in the text to discs A, B, & C, respectively. In each drawing, the upper part shows the disc face, and the lower part shows the cross section of the disc, (the plane of section is indicated in the upper part).


a
a´
a´
a
A
b
b´
b´
b
B
c
c´
c´
c
C

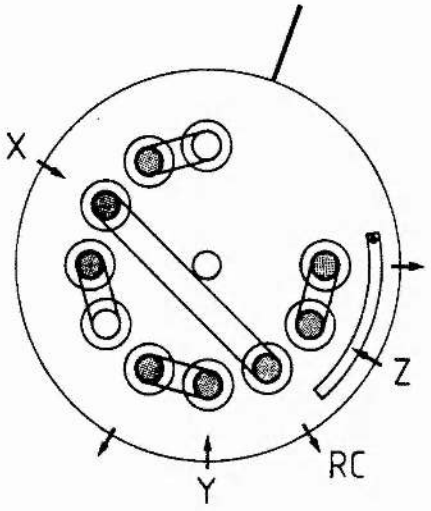
2.2C The Three Way Tap.

The tap was based on an earlier design for a two way tap (Spindler, 1979): its design allowed one of three solutions to flow to the recording chamber, while the other two flowed to waste.

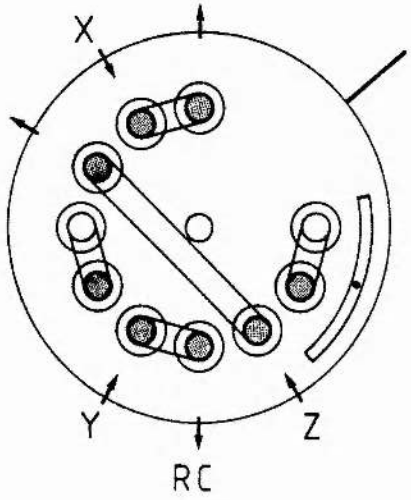
The tap consisted of three discs which formed a cylinder (length 30mm, diameter 40mm): disc A was made of Teflon and discs B & C were made of perspex. For easier description, each disc is considered as a clock face. Disc A (Fig 2.2A) had a hole drilled through the face at 4, 5, 6, 7, 8, 10, 11, and 12 o'clock; the diameter of these holes (1.77mm) was sufficient for polythene tubing to be inserted into each hole for entry and exit of solutions to the tap mechanism. A metal rod was fitted to the edge of the disc as a handle. Disc B (Fig. 2.2B) also had a hole drilled through the face at 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 o'clock; these holes were countersunk and an "O-ring" was inserted into each. Disc C had holes drilled into the face and these holes were joined in pairs by recessed grooves in the face. The holes were paired: 3 & 4, 5 & 10, 6 & 7, 8 & 9, and 11 & 12 (Fig. 2.2C).

Discs B and C were screwed together so that their faces were superimposed with the face of disc C against the reverse of disc B. Disc A was then bolted to the cylinder formed by discs B and C so that the faces of all three discs were superimposed with the face of disc B against the reverse of disc A. The complete cylinder was then bolted to a stand.

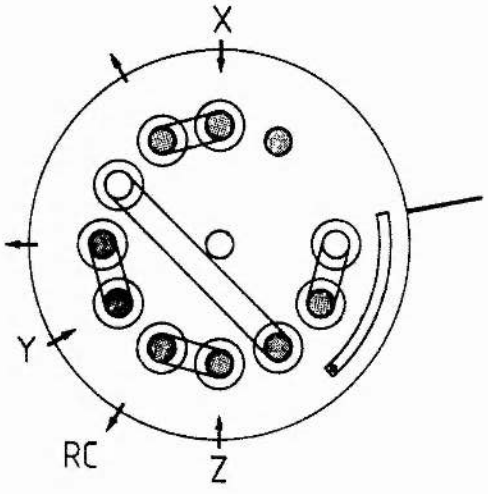
Fig. 2.3 The three discs forming the three way tap drawn superimposed to show the position of each disc in the three tap positions. Discs B & C remain stationary, but disc A, the holes through which are shaded, may rotate from the origin (0^{o}) to the left ($L30^{o}$) or to the right ($R30^{o}$). In each position, one of three solutions (X, Y, and Z) flows to the recording chamber (RC), and the others flow to waste.



L 30°



0°



R 30°

Disc A could now be rotated against disc B. This rotation was limited to 30^{o} in each direction from the origin by a simple stop mechanism which consisted of a screw though disc A which fitted into a groove in the face of disc B. At the two end points and the mid point of the rotation, holes in disc A coincided with holes in disc B so that liquid entering disc A would flow through the tap mechanism. In each position of disc A, a different solution was directed to the recording chamber (Fig. 2.3).

With this tap, changes between one source of ACSF and another could usually be made without interruption to the recordings. However if the tap mechanism was the point of greatest resistance to ACSF flow to the chamber, the tap was less easy to use.

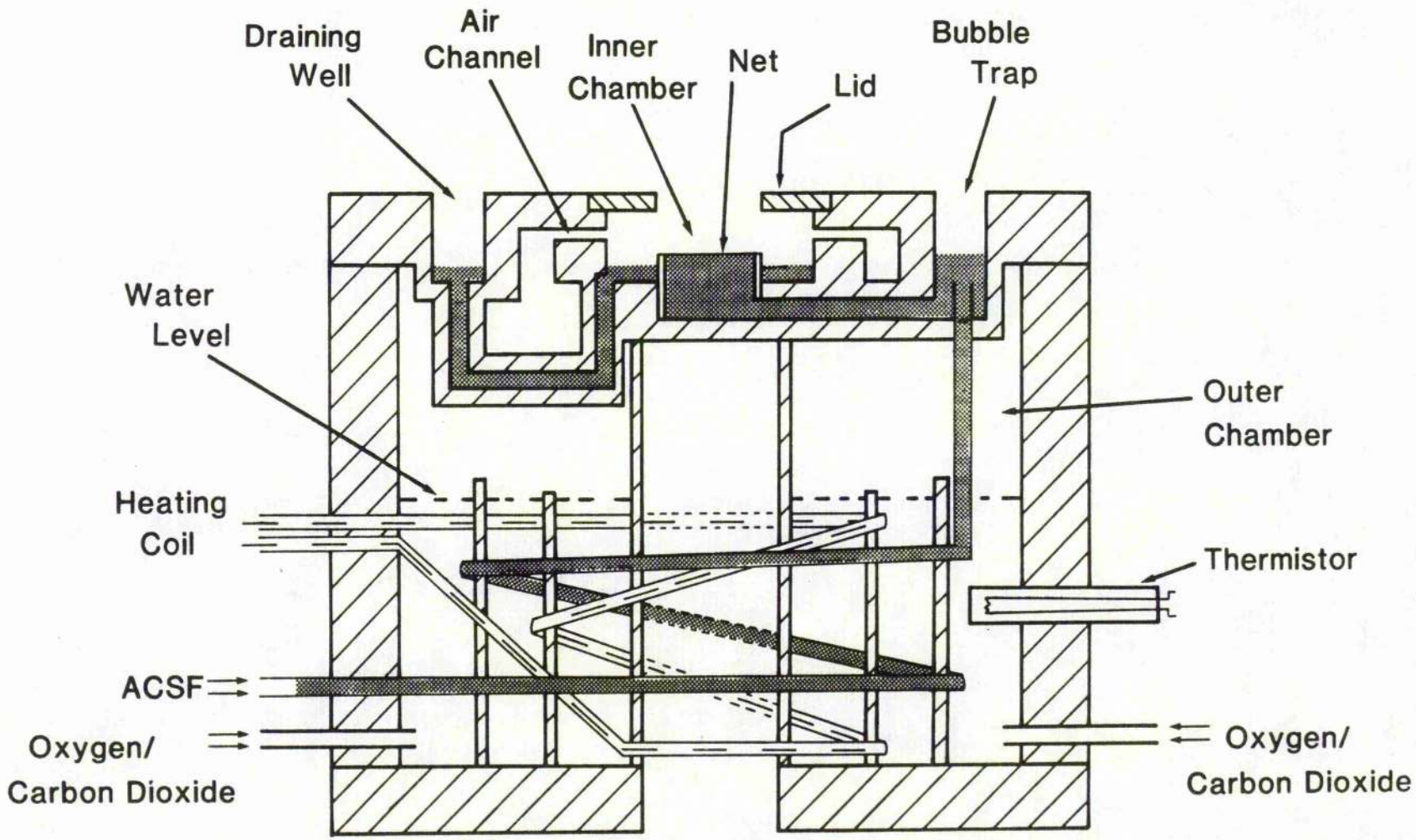
2.3 THE RECORDING CHAMBER

The chamber used was very similar to that used by Skrede & Westgaard (1971), (P.Andersen, personal communication to G.A.Cottrell).

The chamber was made of Perspex and consisted of two concentric tubes which were fixed to a base and which supported a lid (Fig. 2.4). The whole structure formed a cylinder of two major parts: the outer chamber formed by the space between the concentric tubes, and the inner chamber formed by a central well in the lid of the cylinder.

The recording chamber was directly illuminated from above and

Fig. 2.4 Diagram of the recording chamber in longitudinal section showing the different parts of the chamber described in the text. All parts have been shown in the same section for convenience.


Draining Well
Air Channel
Inner Chamber
Net
Lid
Bubble Trap
Water Level
Outer Chamber
Heating Coil
Thermistor
ACSF
Oxygen/ Carbon Dioxide
Oxygen/ Carbon Dioxide

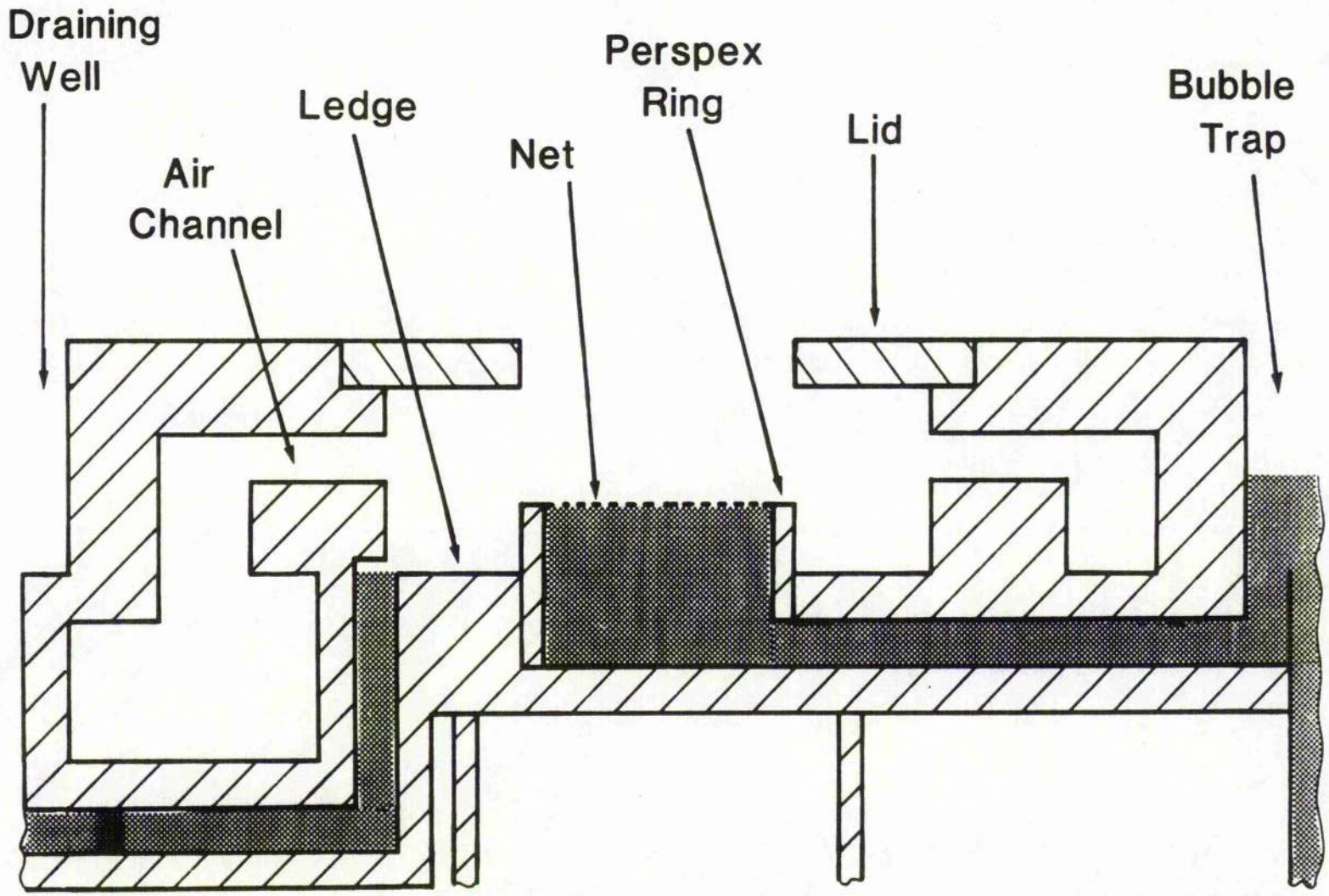
the inner chamber could be viewed with a dissecting microscope (Nikon). The chamber, microscope and light source were mounted on a steel plate which was set on a concrete slab. To prevent transmission of vibration between the slab and the plate, a block of polythene foam was placed under each corner of the plate. The micromanipulators on which electrodes were mounted were also positioned on the steel plate. A Faraday cage mounted on the concrete slab was used to electrically shield the recording chamber.

2.3A The Outer Chamber.

The outer chamber, which acted as a water jacket to the inner chamber in the lid, was filled to a set level with distilled or tap water, (see Fig. 2.4). The water was heated by an insulated resistance wire coiled around a support ring. The temperature of the water was monitored using a thermistor connected to a monitor/control unit (C.J.Rommele, Gatty Marine Laboratory). This unit was connected to and controlled the output of a power source (Farnell Instruments) which supplied current to the resistance wire in the chamber. The system controlled the temperature of the water in the outer chamber to within 1°C of the set temperature.

In the first experiments, the temperature of the water in the outer chamber was set at 37°C. Subsequently it was found that the condition of the slices was improved when the temperature was reduced to 34°C.

Fig. 2.5 Diagram of the inner chamber and the surrounding parts of the recording chamber in longitudinal section. ACSF and the mixture of oxygen and carbon dioxide enter the inner chamber through the bubble trap and the air channels respectively.


Draining
Well
Ledge
Perspex
Ring
Lid
Bubble
Trap
Air
Channel
Net

ACSF and a mixture of oxygen and carbon dioxide (95%:5%) entering the recording chamber were warmed in the outer chamber. ACSF flowed through the chamber in polythene tubing which made two or three turns around the inside of the chamber. The gas mixture was simply bubbled through the water in the outer chamber so that it was simultaneously warmed and moistened. After passing through the outer chamber the ACSF and the gas mixture passed separately to the inner chamber.

2.3B The Inner Chamber.

The inner chamber consisted of a central well in the lid of the recording chamber and its major feature was a nylon net on which the slices were placed (Fig. 2.5). The net was stuck to a Perspex ring (outer diameter 25mm, inner diameter 22mm, and height 7mm). This support ring fitted into the base of the inner chamber so that the base of the inner chamber formed a ledge about 4mm below the net. At first, a net with 1mm mesh was used. Later a net of stretched nylon stocking was found to be more suitable: the mesh size of the stretched stocking was about 0.3mm.

From the outer chamber, ACSF flowed via a bubble trap to emerge in the inner chamber underneath the net. ACSF then flowed up around the net, over the edge of the support ring onto the ledge below, and into a draining well. ACSF was removed from the draining well by suction through a syringe needle which was held at a preset height in the well. The oxygen-carbon dioxide mixture which was warmed and moistened in the outer chamber passed into the

inner chamber through air channels which directed the gas mixture over the nylon net.

A Perspex disc, with a hole in its centre, formed the lid of the inner chamber. The hole gave access to the inner chamber during experiments, but for general access to the inner chamber, the lid was easily removed.

The depth of ACSF on the nylon net was controlled by two major factors. First, the rate of flow of ACSF into the chamber: with faster flow rates (up to 7ml/min) the depth was greater than with slower flow rates. Second, the level of ACSF on the ledge surrounding the net which was determined by the position of the suction needle in the draining well. When the needle was held at the base of the well, ACSF formed a meniscus over the net. The depth of ACSF on the net was reduced by raising the needle in the well, but this change resulted in tidal movements of liquid on the net. The level of ACSF on the ledge was therefore raised artificially by placing a perspex disc on the ledge around the support ring. This disc reduced the depth of ACSF on the net but tidal movements of ACSF on the net were not observed.

2.4 PREPARATION AND MAINTAINANCE OF SLICES

2.4A Preparation of Slices.

In all experiments, slices were prepared from the dorsal part of the right hippocampus of male Wistar rats. The rats weighed

between 130g and 250g and were 30 to 50 days old. When rats smaller than 130g were used, the brain was often badly damaged when it was removed from the skull cavity. However, the thick skull of rats larger than 250g prevented rapid removal of the brain.

Preparation of slices was divided into two phases: i) preparation of the tissue, and ii) slicing of the tissue. After sacrificing an animal, between 2.5min and 5min were required to produce the first useable slice. The last slice was cut not more than 12min after animal death.

i) Preparation of Tissue. The animals were sacrificed by a blow to the neck or thorax. The skin over the skull was cut down the midline, beginning above the neck, and was pinned aside. The skull was opened by cutting through the bone immediately dorsal to the foramen magnum. This opening was extended by cutting forward through the skull, just to the left of the midline, as far as the underlying olfactory bulb. The top of the skull was then peeled away from the midline and broken off with forceps. If present, the underlying dura was then cut away: on many occasions, the dura was removed when the skull was peeled away. A spatula, inserted behind the olfactory bulb, was used to lift the brain out of the skull cavity. Although the cerebellum was occasionally left in the skull cavity, the whole brain except the olfactory bulb was removed using this technique.

After washing in ACSF at room temperature, the brain was placed upright on wet filter paper. The forebrain area, rostral to

the level of the septal nuclei, and the cerebellum were cut away with a razor blade. Two parasagittal cuts were then made: the first was about 2mm from the lateral border of the right hemisphere, and the second was about 1.5mm to the right of the midline. The resulting tissue block formed by the major part of the right hemisphere was placed medial surface uppermost on an aluminium block (10mm × 10mm × 15mm). A few drops of a rapid setting adhesive (Locktite 495) previously poured onto the block ensured that tissue was well stuck to the block within a few seconds. Finally, the neocortex, dorsal to the hippocampus, was cut away. The block of brain tissue mounted on the aluminum block was then placed in the Vibratome chamber.

ii) Slicing of the Tissue. A Vibratome (Oxford Instruments) was used to slice the hippocampus while the tissue was completely immersed in warmed and well-oxygenated ACSF. The ACSF was warmed by water which was heated to a preset temperature and pumped through a heat exchange coil in the Vibratome chamber. The temperature of ACSF in the Vibratome chamber was 37°C in the first attempts to cut hippocampal slices. However better slices were produced when the set temperature was reduced to 34°C. A mixture of oxygen and carbon dioxide (95%:5%) was bubbled through the ACSF to raise the oxygen concentration and to maintain the pH at 7.35.

The aluminum block, on which the tissue was mounted, was placed in the screw clamp in the bottom of the Vibratome chamber. The tissue was cut with a razor blade (Gillette Platinum) which was

mounted so that its height was constant but so that its angle was adjustable. The blade angle was set at 25° below the horizontal in the first attempts to cut slices. In most experiments the angle was between 5° and 10° as better slices were produced with these lower blade angles. The maximum vibrational movement of the blade was usually set. The rate of forward movement of the blade was set at about 10mm/min, but was reduced if the tissue was being compressed rather than being cut by the advancing blade. This problem was most common when cutting through more heavily myelinated regions of the tissue block.

The first slice cut from the tissue block was of unknown thickness and was always discarded. Three or four slices between 300um and 600um thick were then cut. After each slice was cut, it was carefully separated from the tissue block: there were usually attachments to the thalamic area, and via the fimbria to other brain areas.

2.4B Maintainance of Slices.

The slices were transferred individually from the Vibratome to the recording chamber using a wide bore suction pipette filled with ACSF. The slices remained in the chamber over two distinctly separate periods: i) The incubation period, in which recordings could not be made, and ii) the post-incubation period, in which good recordings could be made.

i) The Incubation Period. In the first experiments, the

slices transferred to the recording chamber were positioned on lens tissue placed on the nylon net. However, the slices tended to float off the lens tissue, and the lens tissue tended to float off the nylon net. The upper surface of each slice became dry, and movements of slices on the net were common. Consequently the general condition of the slices was poor: few penetrations of neurones could be made, and these could not be maintained for more than a few minutes.

Several changes in technique were made to improve the condition of the slices and the impalements. The original net was replaced by a net made of nylon stocking, and the slices were placed directly onto this net. In addition, the ACSF level on the net was increased so that the slices were completely immersed in ACSF. The slices were positioned on the net with a fine brush and were fixed in place by pushing extraneous pieces of tissue through the net mesh with a fine needle. Great care was taken to prevent damage to the slices which were always positioned on the net lateral surface uppermost. Once the slices were positioned and fixed in place, the rate of flow of oxygen and carbon dioxide passed over the slices was increased so that the ACSF around the slices was well mixed.

ii) The Post-Incubation Period. When the slices had been in the chamber for about two hours, conditions in the inner chamber were changed. First, air bubbles trapped underneath the net were removed using a syringe fitted with a fine needle. The level of ACSF on the net was then reduced (see Chapter 2.3B) so that the

slices lay more firmly on the net. Lastly, the rate of flow of oxygen and carbon dioxide over the slices was reduced.

Only the lower surface of each slice was then in direct contact with ACSF. The top surface of each slice was covered only by a thin film of ACSF. The thickness of this film was dependent on the humidity of the inner chamber which was dependent on the rate of flow of oxygen and carbon dioxide into the inner chamber. If the slices began to dry out, the rate of flow of the gas mixture was reduced and, if necessary, the upper surface of each slice was moistened using a fine brush.

2.5 RECORDING TECHNIQUES

2.5A Recording Electrodes.

At first electrodes were made from capillary glass with an outer diameter 1.5mm and an inner diameter 0.89mm (Clark Electromedical). The electrodes were pulled using a Livingston style puller (Clark Electromedical). Although the tip impedance was frequently above 50MΩ , these electrodes were not satisfactory: the electrodes would not pass current without noise or capacitance effects. In addition the length of the electrode shank was over 16mm and consequently the electrodes were easily broken. Subsequently, the electrodes were pulled from capillary glass with a larger internal diameter (1.17mm) but the same external diameter. The tip impedance of these electrodes was between 10MΩ and 60MΩ and the electrodes passed current without

noise or capacitance effects. Presumably these "thin" walled electrodes had a greater internal tip diameter than electrodes made from "thick" walled glass.

After filling with filtered electrolyte solution (Millepore, 0.22um filters), the electrodes were inspected microscopically. Electrodes were rejected if poorly shaped, if the tip diameter appeared too large, or if the shank contained air bubbles. Since optical distortions were observed when filled electrodes were inspected, electrodes were frequently inspected before being filled. At first, electrodes were filled with 2M potassium chloride but poor recordings were obtained with these electrodes. Satisfactory recordings were obtained with electrodes filled with 1M potassium acetate. All electrodes were used within two hours of being pulled or were discarded.

2.5B Recording Apparatus.

Conventional techniques were used to amplify, display and permanently record intracellular electrode signals. Electrode signals were amplified (gain x 100) by a high impedance d.c. preamplifier (Digitimer, Neurolog NL102). To reduce junction potentials, chloridised silver wire was used to connect the preamplifier input and reference leads to the solution in the recording electrode and to the ACSF in the recording chamber respectively. An adjustable bridge balance system in the preamplifier allowed simultaneous voltage recording and current injection through the same electrode. Steady injection currents

could be set with a current source within the preamplifier. Current pulses could be simultaneously injected via the the preamplifier from a square wave pulse generator (Tektronix, Model 161) driven by a waveform generator (Tektronix, Model 162). During current injection, the bridge balance was always carefully monitored and was adjusted as necessary to balance out the electrode resistance. The preamplifier signal was amplified and displayed on an oscilloscope (Tektronix 502A).

Permanent records of the oscilloscope signal were usually made with a pen recorder (Gould, Brush 220). However, for permanent records of fast events, the oscilloscope signal was displayed on a storage oscilloscope (Tektronix 564) and the stored events were photographed. The storage oscilloscope was only used to record fast events such as a single action potential since these signals were distorted by the pen recorder.

2.5C Penetration of Neurones.

The recording electrode was mounted on an hydraulic micromanipulator (Stanton, 1976). With this manipulator, fine longitudinal movements of the electrode were made with a remote control.

To examine the condition of a slice, the electrode tip was slowly advanced through the stratum pyramidale of the CA1 region. In healthy slices, positive and negative potential changes were frequently observed as the electrode was advanced (see

Chapter 3.1). Some impalements could be made by advancing the electrode until a negative potential was observed. However, low resting potentials were recorded from neurones penetrated in this way, and impalements could not be maintained for more than a few minutes. Neurones were more satisfactorily penetrated by first advancing the electrode through the stratum pyramidale until a positive potential change was observed. A current pulse was then passed through the electrode to obtain penetration of the neurone. The current intensity was between +30nA and +100nA, and the pulse lasted less than 100ms. Neurones penetrated in this way had satisfactory resting potentials, and the impalements could be maintained for several hours (see Chapter 3).

2.6 APPLICATION OF DRUGS

Agonists were applied by iontophoresis from single micropipette electrodes. The drugs were dissolved in distilled water and, if necessary, the pH of the solution was adjusted. The following drugs were applied by iontophoresis: serotonin oxalate (Sigma) 7.5mg/ml -20mg/ml, pH 4.0 -6.0; serotonin creatinine sulphate (Sigma) 7.5mg/ml - 20mg/ml, pH 4.0 - 6.0; sodium glutamate (Sigma) 200mg/ml, pH 8.0; acetylcholine chloride (BDH) 100mg/ml, pH 4.0.

The iontophoresis electrodes were made on a vertical puller (C.J.Rommele, Gatty Marine Laboratory) from capillary glass (Clark Electromedical): the outer diameter of the glass tubing was 1.5mm and the inner diameter was 1.17mm or 0.89mm. The electrodes had a

short shank (about 6mm) and had tip resistances in the range 5MΩ to 20MΩ . The electrodes were filled with filtered drug solution and were inspected microscopically. Electrodes were not used if there were any air bubbles in the shank. The iontophoretic electrodes were mounted on a micromanipulator (Narashige) for positioning the tip in the slices.

In the first experiments, a simple iontophoresis current control unit was used. However this unit was not wholly satisfactory, and in most experiments an iontophoresis programmer (WP Instruments) was used. Whichever unit was used, the output lead was connected to the drug solution in the electrode via a chloridised silver wire.

Putative antagonists were applied to the slices after being dissolved in ACSF. The drugs were dissolved in 1ml of distilled water or in 1ml 100% ethanol and diluted to the required concentration in ACSF. The following drugs were applied in this manner: atropine sulphate (Sigma) 50μM; cyproheptadine hydrochloride (Merck) 100μM; methergoline (Pharmitalia) 100μM; methysergide bimalienate (Sandoz) 50μM or 100μM; mianserin hydrochloride (Organon) 100μM; l-propranolol (ICI) 50μM or 100μM. Tetraethyl ammonium (TEA) bromide (BDH) 1mg/ml was applied in the same way.

2.7 ANATOMICAL TECHNIQUES

To examine the structure of the neurones in slices, some

neurones were injected with the dye Lucifer Yellow CH (Stewart, 1978). Injected neurones were in slices which were prepared normally and in satisfactory impalements with potassium acetate filled electrodes had been achieved.

Initial attempts to use aqueous solutions of Lucifer Yellow in electrodes produced electrodes which had impedances over 100MΩ, and which would not pass current. Electrodes were therefore filled with with 3% Lucifer Yellow (W/V) in 0.25M or 0.5M LiCl solution. (The LiCl solution was filtered before the Lucifer Yellow was added.) These electrodes had impedances in the range 20MΩ to 60MΩ and passed current satisfactorily.

After penetrating a neurone in the normal way, dye was injected by passing a steady hyperpolarising current of 0.5nA to 2.0nA. The injection period lasted between 2min and 12min, and after an injection was completed the electrode was withdrawn from the slice. Only one attempt to fill a neurone with dye was made on each electrode track through the slice, and only one satisfactory injection was made in each region of a slice. Satisfactory injections were judged as the amount of dye injected estimated from the duration of injection and the injection current. Up to nine successful injections were made in a single session: three injections in each of three slices. Therefore after an injection was made, there was a period of between 15min and two hours for the dye to spread through the neuronal processes. This interval, the post injection incubation period, was considered long enough for complete spread of the dye in the neurone.

During the injections, the laboratory lighting and the direct illumination of the slices were reduced. These precautions were taken since Lucifer Yellow has been reported to be toxic to injected neurones in light (Miller & Selverston, 1979).

After the injections were completed, the slices were processed using methods described by Murphy & Kater (1980) for examination of snail neurones injected with Lucifer Yellow. Each slice was floated off the net and transferred using a wide bore suction pipette to a solution of 4% formaldehyde in 0.1M phosphate buffer, pH 7.4, for between 16hrs and 24hrs. During this period the slices were kept at $4^{o}C$ in the dark. Slices were dehydrated in an ascending ethanol series, and cleared and mounted in methylsalicylate.

Intact slices were examined on a Leitz Ortholux microscope. For general examination the slices were transilluminated with normal light and for fluorescence microscopy incident illumination with ultra-violet (U.V.) light was used. The microscope was fitted with an HB 200 mercury lamp, an incident light Ploem condensor, a 5mm BG12 excitation filter, and with K460 and K510 barrier filters. The microscope was also fitted with a tungsten light source for normal light microscopy. The arrangement of the two light sources allowed simultaneous fluorescence and normal light microscopy.

Black and white photographs were taken using Tri-X Pan negative film (Kodak, ASA 400): exposure times for fluorescence fields were between 8s and 12s. Color photographs were taken using

CT21 transparency film (Agfa, ASA 100): exposure times were 18s to 22s.

CHAPTER THREE

RESULTS I

GENERAL PROPERTIES OF

CA1 HIPPOCAMPAL NEURONES.

3.1 EVALUATION OF SLICES.

The general condition of slices was examined before attempts to make long lasting penetrations of neurones were made. The examination was made by observing the frequency and amplitude of two types of potential change recorded as the tip of the electrode was advanced slowly through the stratum pyramidale of the CA1 region. The first type of change was a positive potential which probably represented the tip of the electrode being advanced against the membrane of a neurone. The second was a negative potential which represented the penetration of a neurone. Since the electrode was continuously moving, these potentials were transient. Using this method, the condition of the slices could rapidly and reliably be evaluated.

The slices were considered to be in good condition if such potential changes were observed frequently, indicating a large number of healthy neurones. The maximum amplitudes of the negative and positive potentials were about -35mV and 10mV in these slices. Only in those slices shown to be in good condition, could satisfactory, long lasting impalements be achieved.

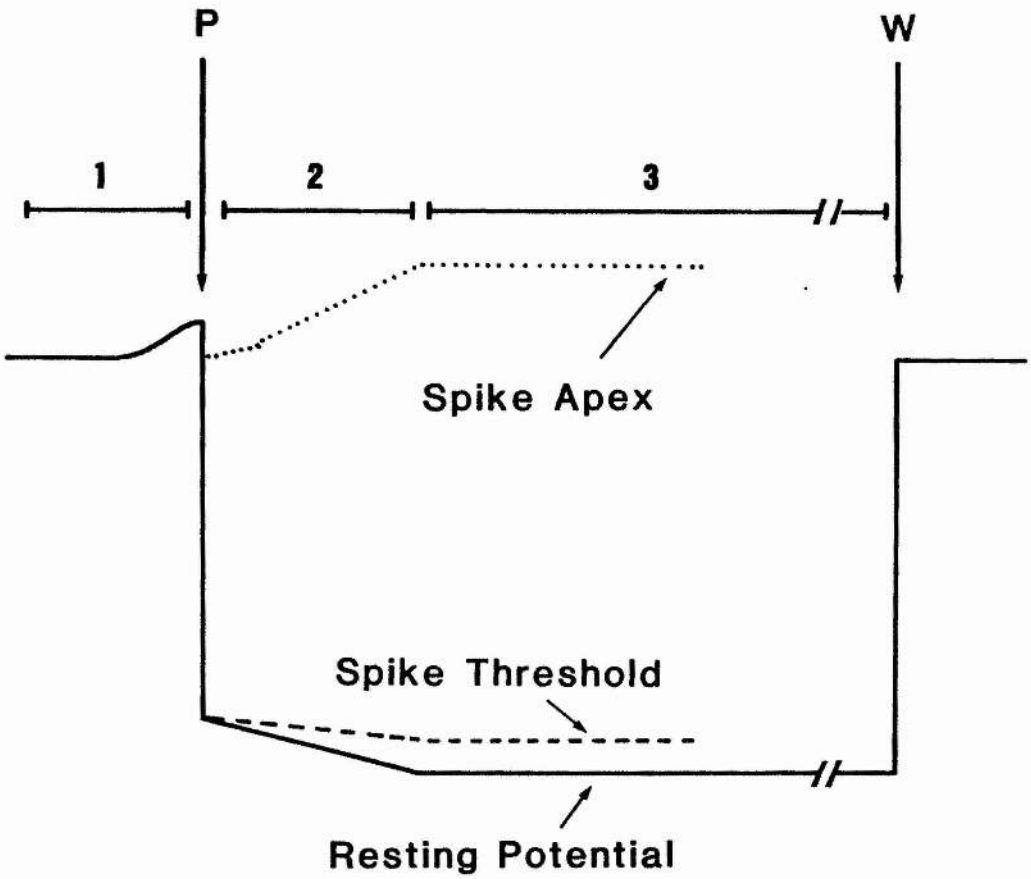
3.2 PROPERTIES OF CA1 NEURONES

The general properties of the CA1 pyramidal neurones in the guinea pig hippocampal slice preparation have been characterised by Schwartzkroin (1975, 1977). Before this study on the effects of

Fig. 3.1 Diagramatic representation of the potential changes before, on, and following penetration of a neurone, and on withdrawal of the electrode. There are three phases, marked 1, 2, & 3.

Phase 1: the electrode is advanced into the tissue until a positive potential is recorded, and then penetration of a neurone is achieved (P) by passing a brief current pulse of between 30nA and 100nA through the electrode.

Phase 2: the resting potential increases to a steady level, and the amplitude of action potentials increases as the spike apex and threshold become more positive and more negative respectively. Phase 3: The amplitudes of the resting and action potentials are constant, and the firing rate is less than 5Hz. When the electrode is withdrawn (W), the potential returns to zero.


P
W
1
2
3
Spike Apex
Spike Threshold
Resting Potential

serotonin on neurones in rat hippocampal slices could be started, it was necessary to obtain routinely long lasting intracellular recordings of a similar quality.

3.2A Penetration of CA1 Neurones.

In the first experiments, neurones were penetrated by slowly advancing the electrode through the stratum pyramidale until a negative potential change was observed. However such penetrations were not satisfactory as resting and action potentials were always less than 50mV. Further, the resting potentials of these neurones decreased, indicating substantial injury to the neurone, and after about ten minutes, the impalements were lost. The poor impalements obtained in these experiments were in part due to the method of penetration. In later experiments, this method was again unsatisfactory, whereas in the same slices, better impalements were achieved with another method of penetration.

Satisfactory impalements were made by passing a brief current pulse through the electrode as described in Chapter 2.5C. On penetration, resting potentials of up to -60mV and spontaneous action potentials of similar amplitude were observed. If the impalement was satisfactory, the resting potential increased during the first ten minutes after penetration. During the same period, the amplitude of spontaneous spikes also increased: the spike apex became more positive and the spike threshold became more negative (see Fig. 3.1). In contrast, the frequency of these spikes decreased from about 30Hz on penetration to less than 5Hz by the

time the resting potential was stabilised. After this period of stabilisation, resting and action potentials were often of constant amplitude for several hours.

The characteristics of impaled neurones were improved when ACSF containing 3.75mM K^+ and 4mM Ca^{++} was used. In addition, the number of satisfactory impalements which could be made was increased in slices bathed in this ACSF. Compared to neurones impaled in slices bathed in ACSF containing 6.25mM K^+ and 2mM Ca^{++}, the following differences were observed. 1) Resting potentials were slightly increased. 2) The frequency of spontaneous action potentials was reduced. In addition, multiple spike discharges superimposed on the resting potential or on depolarising potential shifts lasting a few seconds were observed less frequently. 3) A clearly defined afterhyperpolarisation of the action potential was observed in all neurones, whereas a depolarising after potential (DAP) had previously been observed in some neurones.

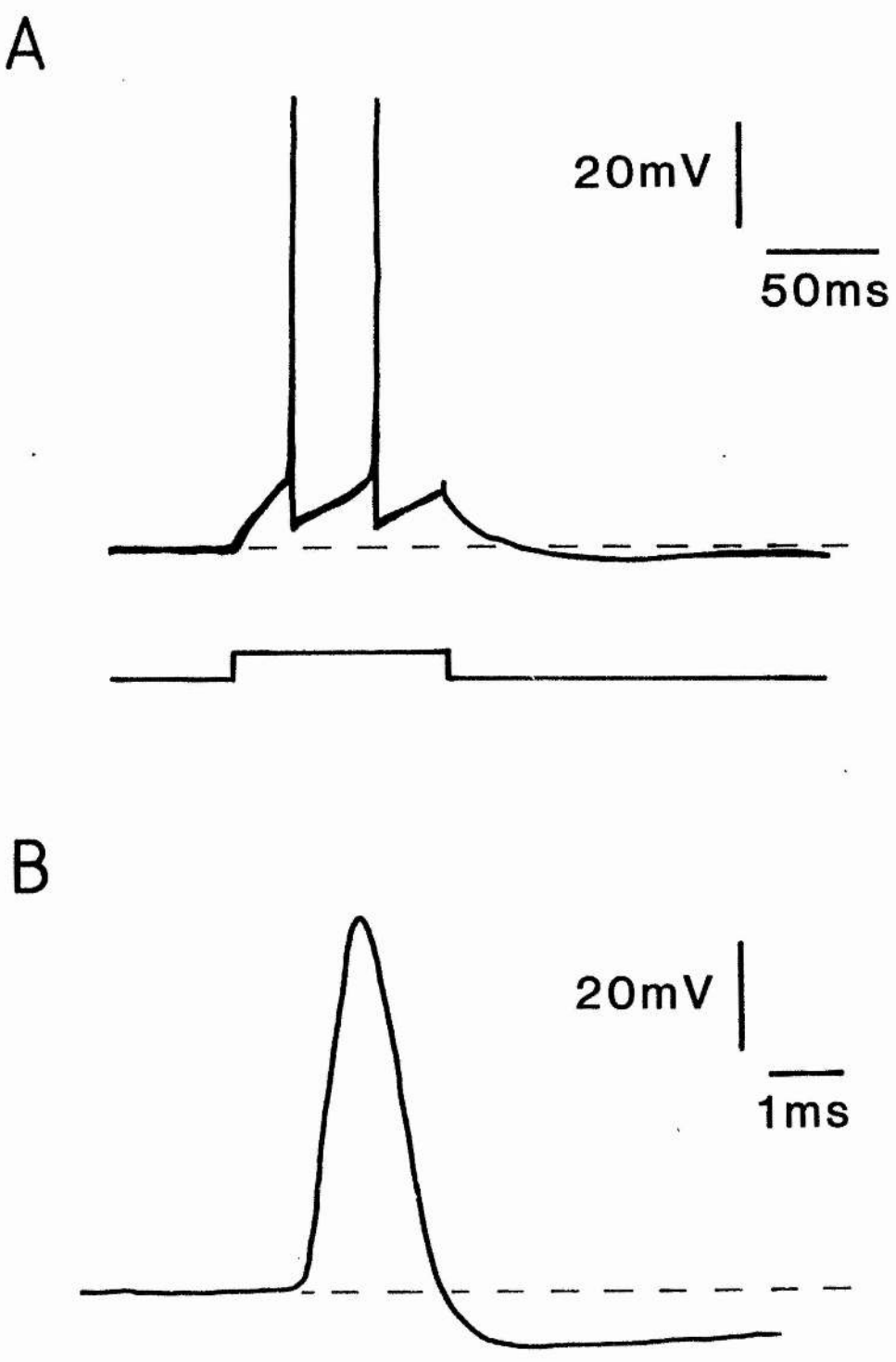
3.2B Resting Potentials.

In neurones impaled for more than 45min, the mean resting potential was -63.4mV (S.D. ±8.7mV) and the range was -45mV to -80mV. The frequency distribution of observed resting potentials indicated that recordings were probably made from a homogeneous population of neurones. The resting potentials and other properties of the neurones were similar to those recorded *in vivo* and *in vitro* by other workers.

Fig. 3.2 Tracings of photographic records from an oscilloscope which were obtained during impalement of a CA1 pyramidal neurone with a recorded resting potential of -65mV.

A: A depolarising current pulse injected intracellularly, lower trace, produced a depolarisation and two action potentials, upper trace. A depolarisation of 12mV was required to trigger the action potentials. The dotted line in the upper trace shows the recorded resting potential.

B: An action potential triggered by a depolarising current pulse recorded at a faster speed. The spike amplitude was 72mV and its duration was 2.0ms. The dotted line represents the potential immediately before the rapid depolarising phase of the action potential and the afterhyperpolarisation can be clearly seen.


A
20mV
50ms
B
20mV
1ms

A slight decline of the resting potential was ~~expected~~ expected during an impalement lasting several hours. The resting potential was therefore measured routinely when the electrode was withdrawn from a neurone as well as on penetration. (Some difference between the potentials at these two times was expected due to difficulties of exact measurement of the potential on penetration.) The resting potentials of most neurones were constant during long lasting impalements, and any reduction of the resting potential during an impalement was gradual and less than 5mV. These small changes were disregarded. If the change was larger, and accompanied by an increase of the spontaneous spike frequency, deterioration of the neurone or the impalement was indicated. The data obtained during such impalements were discarded.

3.2C Action Potentials.

The mean amplitude of action potentials of satisfactorily impaled neurones was 72.5mV (S.D. ±8.0mV) (see Fig. 3.2), and the range was 45mV to 88mV. Spontaneous spikes, that is impulses that were not evoked by electrical stimulation or by application of drugs, were observed to be preceded by an excitatory postsynaptic potential (e.p.s.p.), or were superimposed on the resting potential in the absence of an observed e.p.s.p.. The frequency of spontaneous spikes was less than 5Hz, and in many neurones, spontaneous spikes were not observed at all after the stabilisation of the resting potential. Anode break spikes were observed in many neurones on the termination of hyperpolarising current.

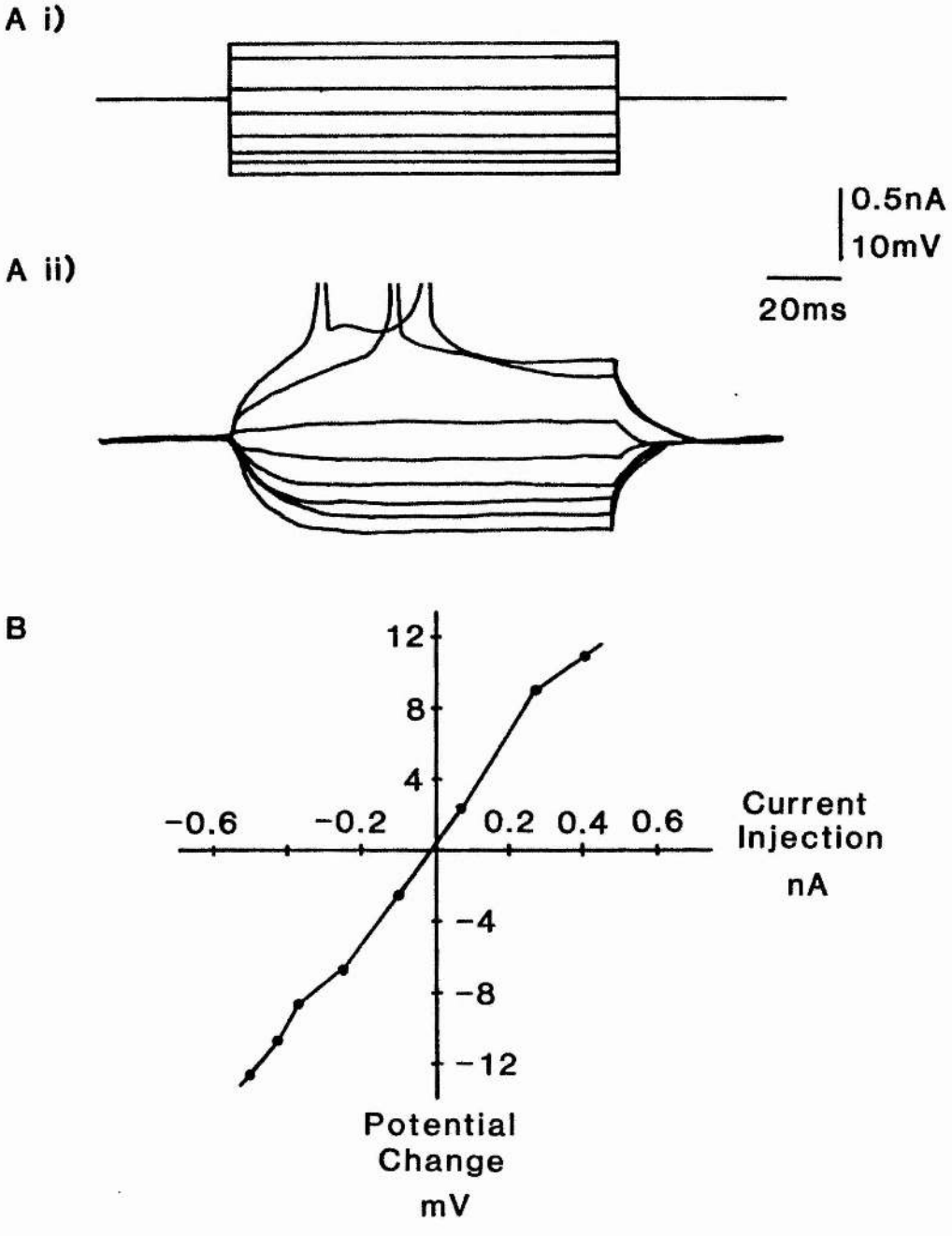
When spontaneous impulses were not observed, a single spike could be generated by injecting depolarising current: between 1mV and 20mV depolarisation was required. Injection of larger currents generated a train of spikes and the frequency of these impulses was dependent on the current intensity.

There was a correlation between the amplitude of the spike and of the resting potential, but not between the spontaneous spike frequency and and the recorded resting potential. Rather, the spontaneous firing rate was dependent on the potential change which occurred during stabilisation of the resting potential after penetration. When this potential change was large, the impulse frequency was low independent of the resting potential.

In some experiments, the spontaneous spike rate changed during a long lasting impalement. Such changes were usually small and were disregarded. If a large increase of the frequency was observed, together with a decreased resting potential, deterioration of the penetration was indicated.

In addition to normal action potentials, transient depolarisations on which a single action potential might be superimposed were observed in some neurones. These depolarisations did not resemble e.p.s.p.'s, and were probably dendritic spikes which did not invade the perikaryon. These "spikes" were only recorded in the first few minutes after penetration.

Fig. 3.3 A: Tracings of photographic records from an oscilloscope of i) a series of intracellular current injections, and ii) the potential changes produced by these current pulses. The resting potential of this CA1 pyramidal neurone was -60mV, and 12mV depolarisation was required to trigger action potentials. B: The relationship between the intensity of the intracellular current injection and the resulting potential change obtained from the records in (A). The sizes of the currents and potential changes were measured 15ms before the termination of the injection pulse. The input resistance of this neurone was 25MΩ, and over the potential range, there was only a small amount of anomalous rectification.


A i)
0.5nA
10mV
A ii)
20ms
B
12
8
4
-0.6
-0.2
0.2
0.4
0.6
Current
Injection
nA
-4
-8
-12
Potential
Change
mV

3.2D Spontaneous Synaptic Potentials.

Spontaneous e.p.s.p.'s and i.p.s.p.'s, were recorded from many neurones. The frequency of these potentials was not dependent on the resting potential. The synaptic potentials were not analysed, but their frequency could be used as a guide to the excitability of the neurones in a slice.

3.2E Input Membrane Resistance.

In many neurones, the input membrane resistance was measured by recording the potential changes resulting from positive and negative current injections. A typical current injection - potential change relationship obtained in these experiments is shown in Fig. 3.3. The mean input membrane resistance was 16.5MΩ . In a few of these neurones, there was evidence of anomalous rectification: as the membrane was hyperpolarised, the input resistance of the membrane was decreased.

3.2F Criteria for Satisfactory Penetrations.

The properties of neurones described above show the characteristics of satisfactorily impaled neurones. Three criteria were used for describing a satisfactory impalement of a neurone: 1) a recorded resting potential of at least -45mV, 2) overshooting action potentials greater than 50mV generated spontaneously or by injecting current, and 3) a spontaneous spike frequency of less than 5Hz. These criteria are similar to those

used by other workers.

3.3 EFFECTS OF EXTRACELLULAR IONIC CHANGES ON THE NEURONES

Two types of experiment were done which demonstrated that the extracellular fluid in the slices was in equilibrium with the ACSF bathing the slices. These experiments also showed how rapidly equilibrium between the extracellular fluid and the ACSF was reached after the composition of ACSF entering the recording chamber was changed. The effects of altering the composition of ACSF on the resting potential, spike frequency and duration, and the spontaneous synaptic frequency were examined.

In the first type of experiment, the effects of raising the potassium concentration in ACSF from 3.75mM to 13.75mM or to 21.25mM were examined. In the second, the effects of tetraethyl ammonium (TEA) ions (1mg/ml) added to ACSF were investigated.

In these experiments, a satisfactory impalement was made when the slices were bathed in normal ACSF. Then, without interruption to the recordings, high K^+- or TEA-ACSF was introduced into the recording chamber. The effects of the ACSF change were observed, and once equilibrium was reached, normal ACSF was re-introduced into the recording chamber. The equilibrium was indicated when no further changes of the properties of the neurone were observed.

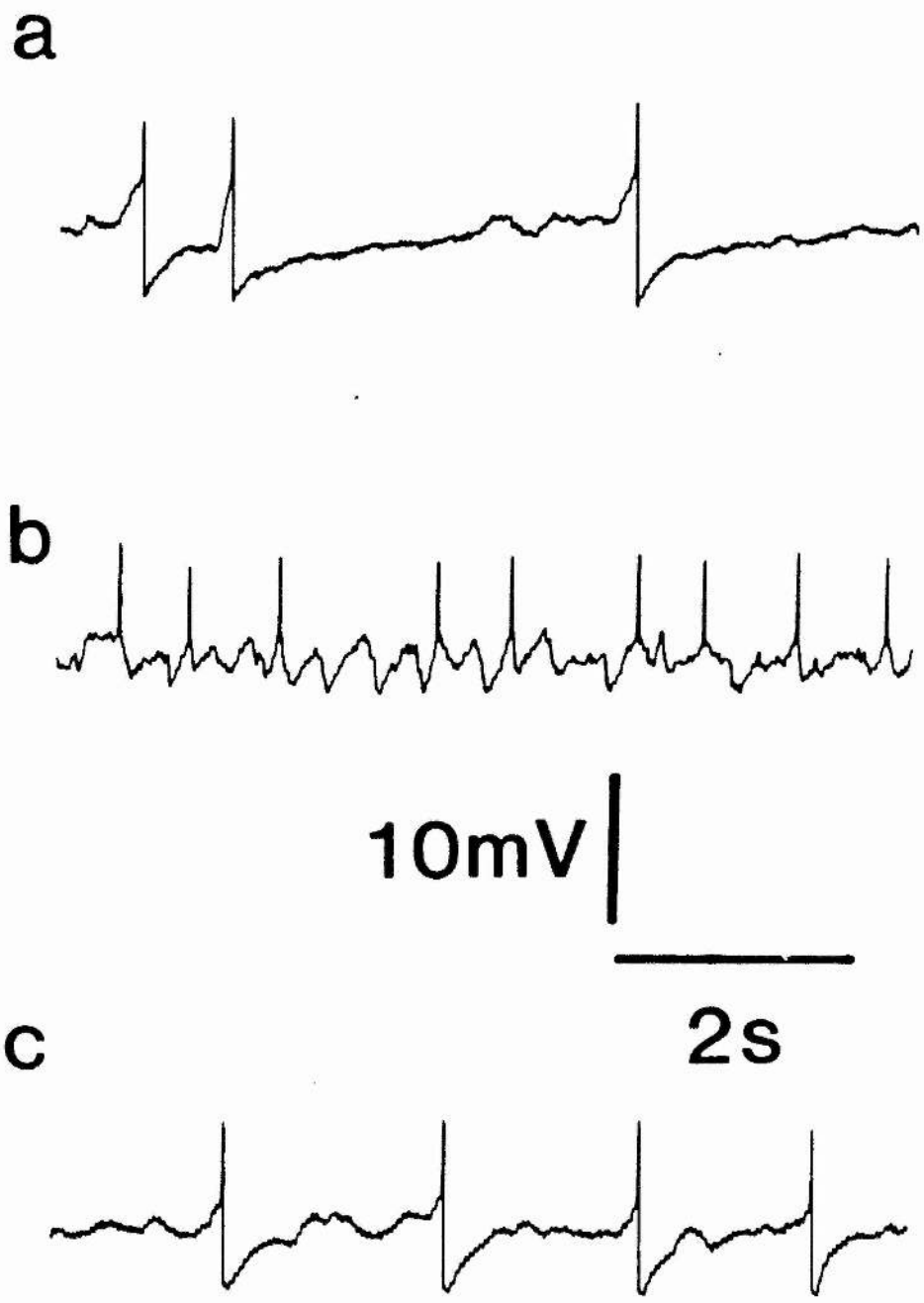
The time course of the effects of raising the K^+ concentration and of adding TEA were similar. After changing the

Fig. 3.4 Records from an experiment made to examine the the effects on a neurone of raising the potassium concentration of ACSF from 3.75mM to 13.75mM. The amplitude of the action potentials in these records have been greatly reduced by the pen recorder.

a: Record made when the K^+ concentration of ACSF was 3.75mM (i.e. normal).

b: Record made 13min after the K^+ concentration in the ACSF was raised to 13.75mM. The firing rate and the frequency of observed spontaneous synaptic potentials were increased, and the neurone was depolarised by 7mV. The increased K^+ concentration also abolished the afterhyperpolarisation of the action potential.

c: 10min after the K^+ concentration in ACSF entering the recording chamber was returned to 3.75mM, this record was made showing that the firing rate and the frequency of synaptic potentials were normal. The normal amplitudes of the resting potential and the spike afterhyperpolarisation were also recorded.


a
b
10mV
2s
c

ACSF entering the chamber, the recordings were unaltered for about two minutes. In the subsequent 5min to 8min, the characteristics of the neurones changed and equilibrium was reached. A total of between 7min and 10min was therefore required to completely change the extracellular fluid.

3.3A Effects of Raised Potassium Concentration.

When the K^+ concentration of ACSF was raised from 3.75mM to 13.75mM, the resting potential was reduced by between 5mV and 10mV. In addition, the spontaneous spike frequency was increased and the afterhyperpolarisation of the spike was decreased (Fig. 3.4). An increased frequency of spontaneous synaptic potentials indicated that the increased K^+ concentration occurred throughout the slices.

When the K^+ concentration of ACSF was raised from 3.75mM to 21.25mM, the resting potential decreased by about 15mV. During the potential change, the spontaneous spike frequency and the frequency of spontaneous synaptic potentials were initially increased. However, at the final potential, spontaneous impulses were apparently suppressed and spontaneous synaptic potentials were not recorded.

When the K^+ concentration was returned to 3.75mM, the resting potential, spike frequency and spontaneous synaptic potential frequency returned to the original values.

3.3B Effects of TEA Ions.

The major effect of TEA (1mg/ml) was an increase of the spike duration. A small depolarisation of up to 5mV, and an increase of the frequency of spontaneous spikes and of synaptic potentials were also observed.

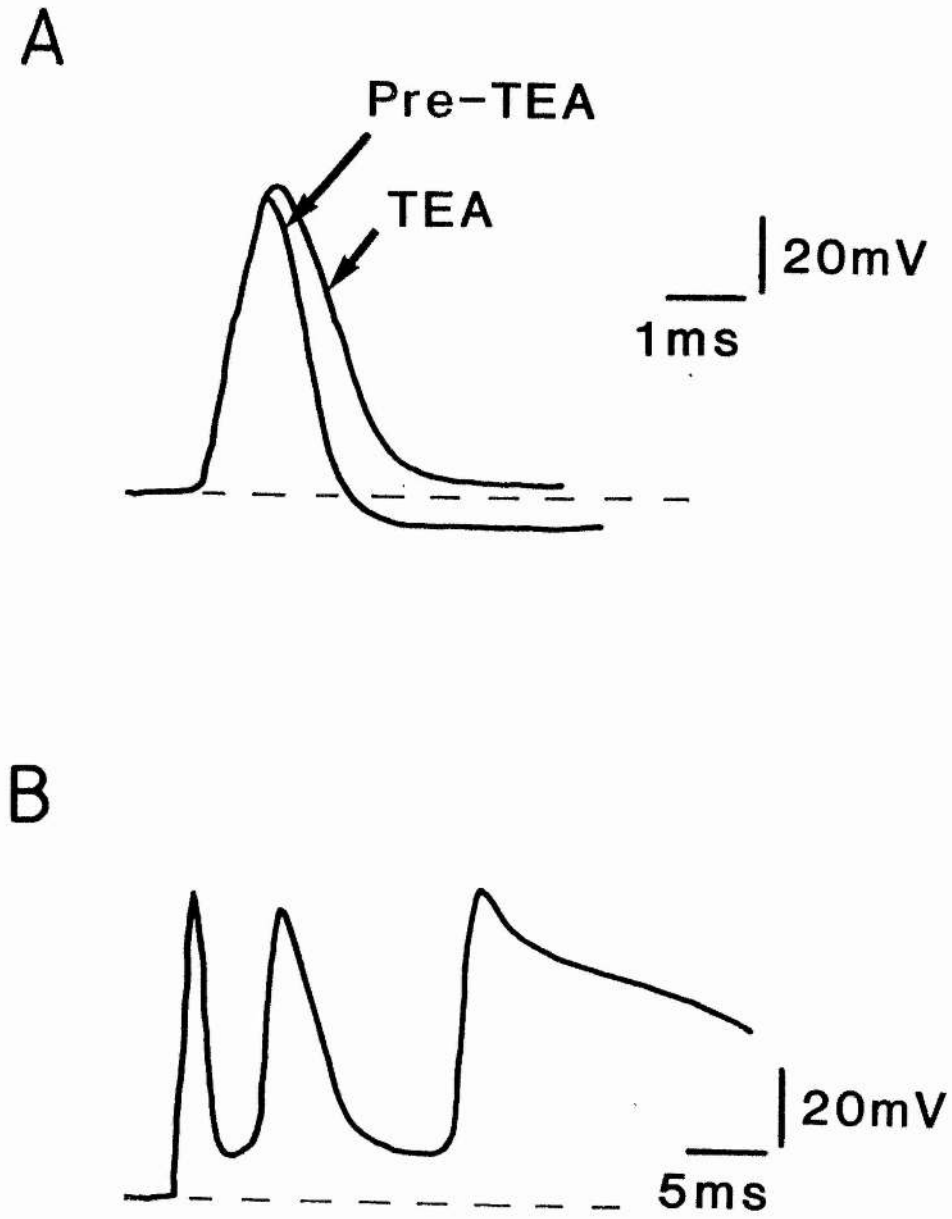
The effect on the duration of action potentials was examined as follows. A satisfactory impalement was made while the slices were bathed in normal ACSF. Current pulses (frequency 1Hz, duration 200ms) were injected so that a single action potential was generated by each pulse. ACSF containing TEA was then introduced into the recording chamber. TEA increased the duration of the action potential from about 1.8ms to about 4.5ms, and abolished the afterhyperpolarisation of the spike (Fig. 3.5A). In addition, a train of spikes was now generated by each current pulse (Fig. 3.5B). The effects of TEA were greater on later spikes of these trains than on the first spike, so that the final action potential in a train was up to 25ms in duration. When normal ACSF was re-introduced to the recording chamber, the spike duration, the afterhyperpolarisation of the action potential, the resting potential, and the frequency of spontaneous synaptic potentials returned to their original values.

Although the effects of TEA on the neurones occurred as rapidly as the effects of raising the potassium concentration, the effects of TEA disappeared more slowly. Reversal of the effects of high K^+ required about 10min, but the effects of TEA were

Fig. 3.5 Tracings of photographic records from an oscilloscope to show the effect of TEA (1mg/ml) on the spike duration. In A & B, the dotted line represents the potential immediately before an action potential was triggered during intracellular injection of current.

A: Comparison of the action potential recorded while the slices were bathed in normal ACSF (Pre-TEA) and the first spike triggered during injection of the same current after addition of TEA to ACSF (Post-TEA). TEA prolonged the action potential and abolished the afterhyperpolarisation of the spike. When normal ACSF was re-introduced into the recording chamber, the effects of TEA were reversed.

B: The first three action potentials triggered by current injection while the slices were bathed in ACSF containing TEA (in normal ACSF only one impulse was triggered). The effects of TEA appeared to increase with each spike during such trains.


A
Pre-TEA
TEA
20mV
1ms
B
20mV
5ms

reversed only after washing the slices with normal ACSF for about 25min.

CHAPTER FOUR

RESULTS II

LUCIFER YELLOW INJECTIONS OF HIPPOCAMPAL NEURONES

4.1 GENERAL OBSERVATIONS

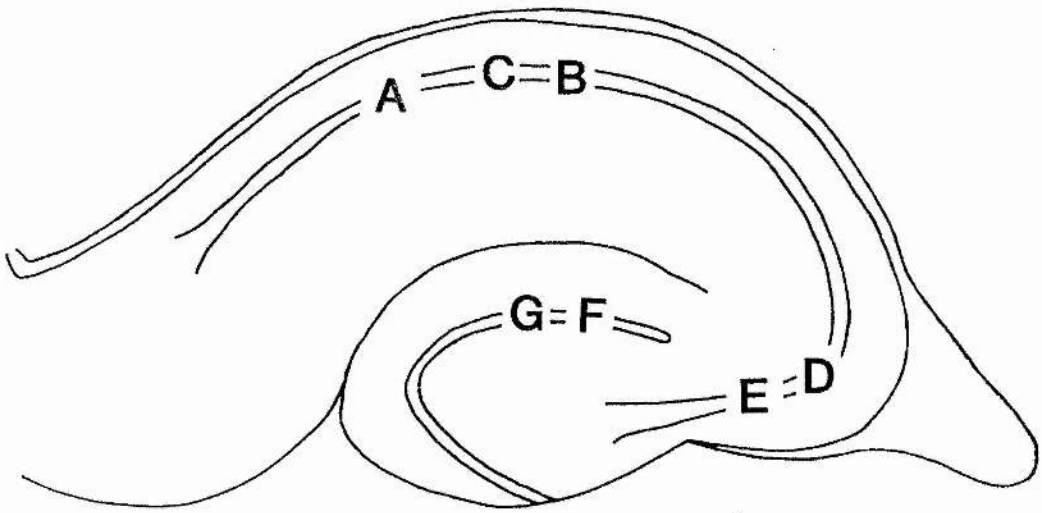
The aim of this series of experiments was to identify and examine the structure of neurones normally impaled in the CA1 region by intracellular injection of the fluorescent dye Lucifer Yellow CH Some injections were also made in the CA3 region and in the area dentata.

Sixty-nine neurones were satisfactorily injected with Lucifer Yellow CH and were subsequently examined microscopically. Neurones containing dye fluoresced bright green against a dark background. Of the 69 injected neurones, 52 were sufficiently filled with dye for photography and later two dimensional reconstruction. The remainder were poorly filled with dye: the fluorescence intensity was low, and only the perikarya and the proximal dendrites of the injected neurone were fluorescent. There was no evidence for leakage of dye from any of the injected neurones, as dye was not observed in the extracellular space.

The intensity of fluorescence and the distance moved by the dye within the processes of injected neurones was independent of the duration of the post injection incubation period which was always more than 15min. Injected neurones were either intensely fluorescent with even distal processes well filled with dye, or weakly fluorescent with only the perikaryon and the proximal dendrites apparently containing dye. In every injected neurone, the perikaryon was always more intensely fluorescent than any other part.

Fig. 4.1 Diagram of the hippocampal formation in transverse section to show the positions and types of the injected neurones which are illustrated in this Chapter. The orientations of the neurones in the subsequent figures are the same as the orientation of this figure.
The positions of the neurone(s) illustrated in each Figure are shown below.

Position	Neurone Type	Figure(s)
A	CA1 Pyramidal	4.2 & 4.3
B	CA1 Pyramidal	4.4
C	CA1 Pyramidal	4.5
D	CA3 Pyramidal	4.8
E	CA3 Pyramidal	4.6 & 4.7
F	Granule	4.9 & 4.10
G	Granule	4.11


A
C
B
G
F
E
D

The intensity of the dye fluorescence decreased during prolonged exposure to U.V. light, but fading per se was not a serious problem. However, tissue autofluorescence increased during the same period. Consequently, after about 25min exposure to U.V. light, the intensity of the tissue autofluorescence was about the same as the fluorescence of many of the dye filled neuronal processes. When slices were kept in the dark at $4^{o}C$, the intensity of the fluorescence of dye filled neurones was maintained for at least four weeks.

The resting potentials of CA1 neurones, and neurones in the CA3 region and area dentata, penetrated with electrodes filled with LiCl/Lucifer Yellow CH solution decreased to between -40mV and -50mV within 30s after penetration. These resting potentials were low compared to those recorded from neurones satisfactorily impaled with potassium acetate filled electrodes, and at these potentials, spontaneous spikes were suppressed. When hyperpolarising current was passed to inject dye, the potential was increased and normal spikes were observed.

The positions of the injected neurones illustrated in photographic montage or after reconstruction in two dimensions in this chapter are shown in Fig. 4.1.

4.2 DYE INJECTIONS IN THE CA1 REGION

From twenty nine injections, 37 neurones in the CA1 region were sufficiently filled with dye to be photographed and

Fig. 4.2 Photographic montage of a CA1 pyramidal neurone injected with Lucifer Yellow. The apical dendrites projected as far as the fissure and the basal dendrites to the alveus. Note that since the dendrites pass into and out of the focal plane, some dendrites may not be shown and some may not appear complete. All the dendrites of this neurone that were observed are shown better in Fig. 4.3.

The marker bar represents 75μm.

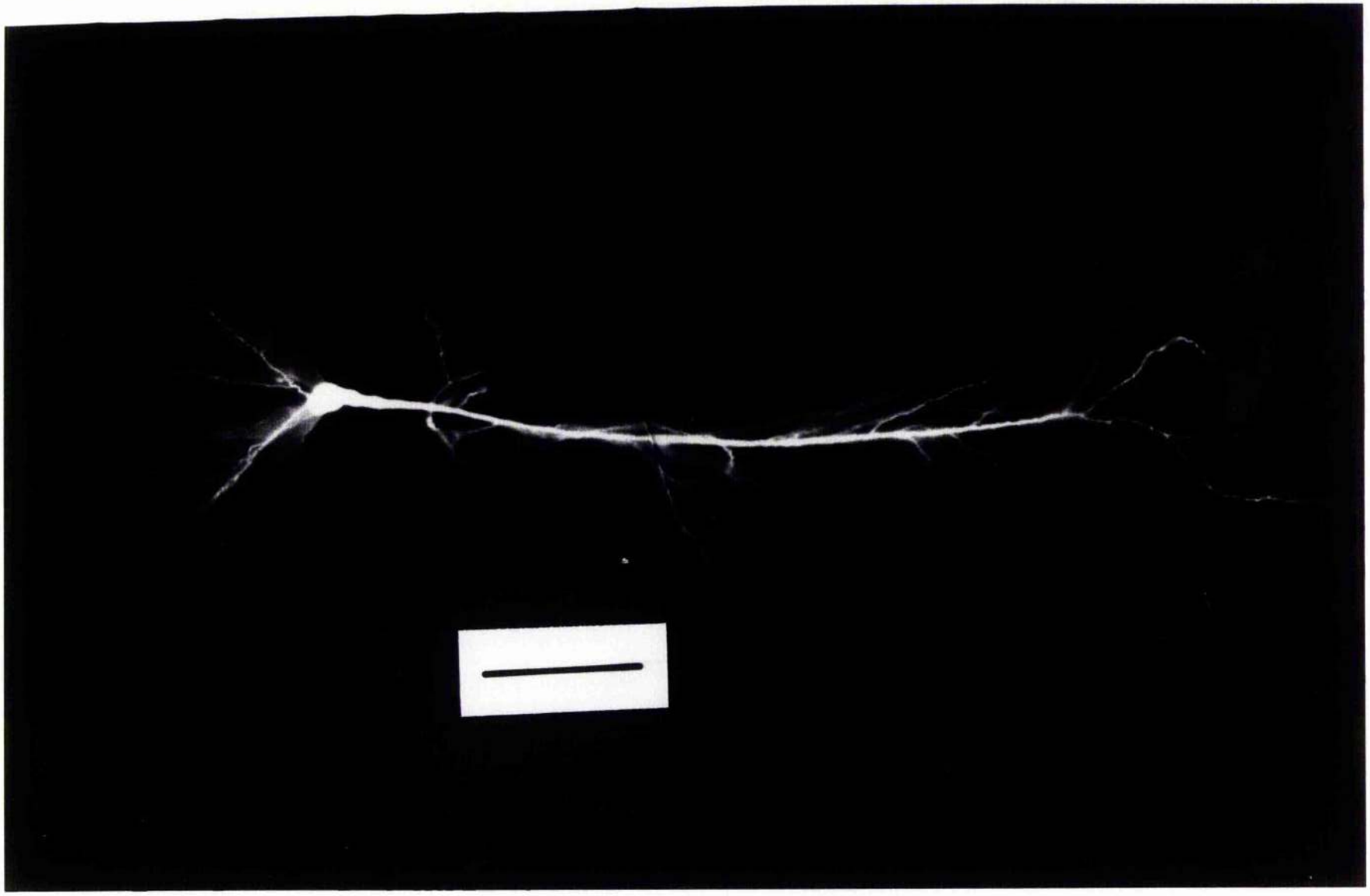
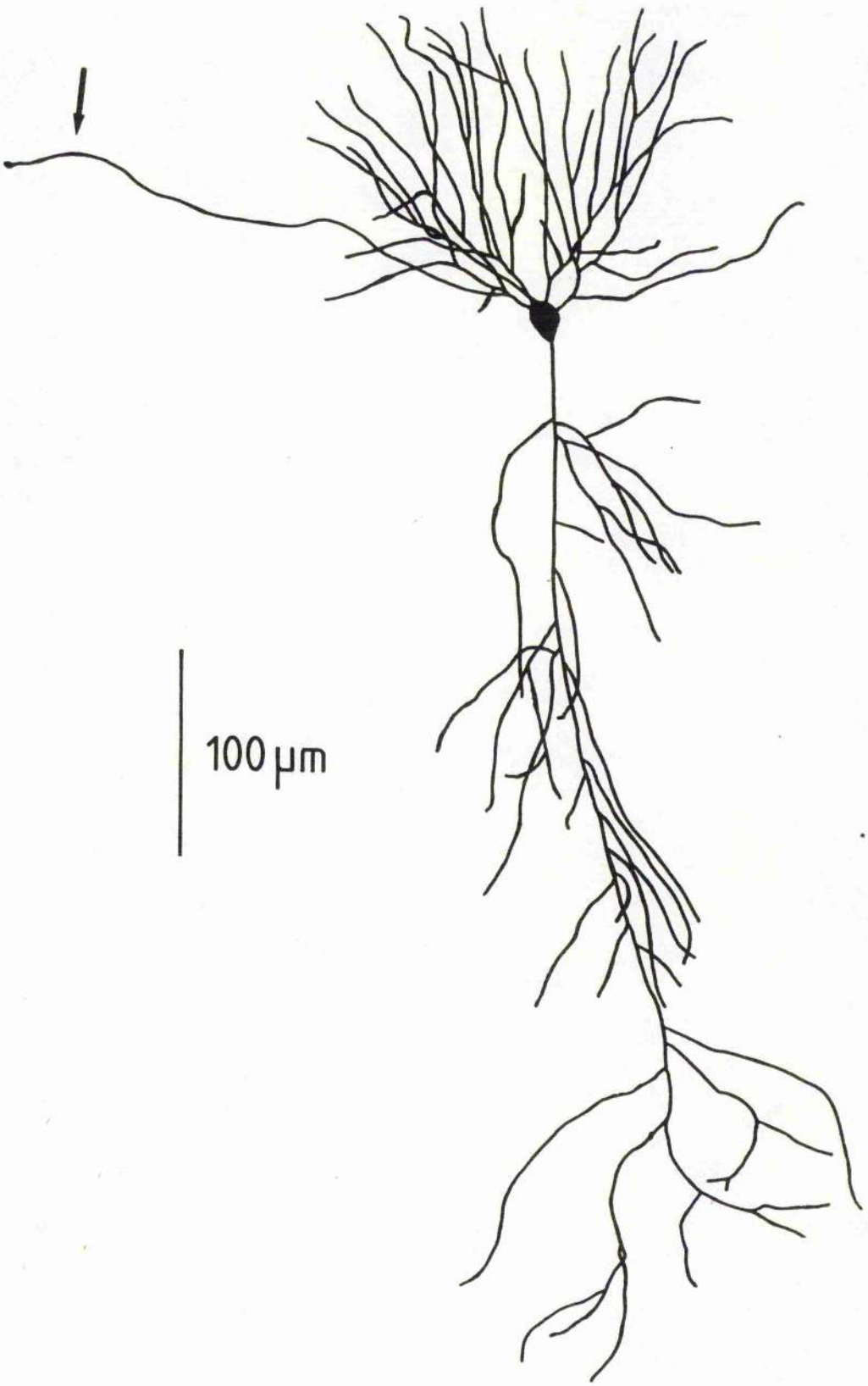


Fig. 4.3 Reconstruction of the neurone shown in Fig. 4.2. The axon (arrowed) was traced for about 300μm until it passed out of the slice. This neurone had a single, "non-dividing" apical dendrite.


100 µm

subsequently reconstructed in two dimensions. The general morphology of the injected neurones was consistent with the previous description of pyramidal neurones in this region from silver impregnated preparations (Cajal, 1911; Lorente de No, 1934; Harris, Cruce, Greenough & Teyler, 1980).

The diameter of the perikarya ranged between 15μm and 25μm as measured perpendicular to the dendritic axis of the injected neurone. Several dendrites were observed to project from the basal surface of the perikarya of injected neurones. These dendrites branched in the stratum oriens and could be traced as far as the alveus (about 150μm). Usually only one dendrite was observed to project from the apical surface of the perikarya. The surfaces of the basal and apical dendrites were smooth along their whole length, and clearly defined dendritic spines were not observed.

No structural differences were observed between those pyramidal neurones with their perikarya deep in the stratum pyramidale and those with their perikarya in the superficial part of the stratum pyramidale. However two types of pyramidal neurone were distinguished on the basis of the branching of the apical dendrite. One type (19 cells) had a single, "non-dividing", apical dendrite extending from the perikaryon to the fissure (Figs. 4.2 & 4.3). The apical dendrite of the second type of neurone (18 cells) divided into two or more major branches within 200um of the perikaryon (Fig. 4.4), and these branches extended as far as the fissure. In both types of neurone, fine, short dendrites branched off the major dendrite(s).

Fig. 4.4 Reconstruction of a CA1 pyramidal neurone injected with Lucifer Yellow. This neurone had an apical dendrite that divided into two major branches about 120μm from the perikaryon (arrow).

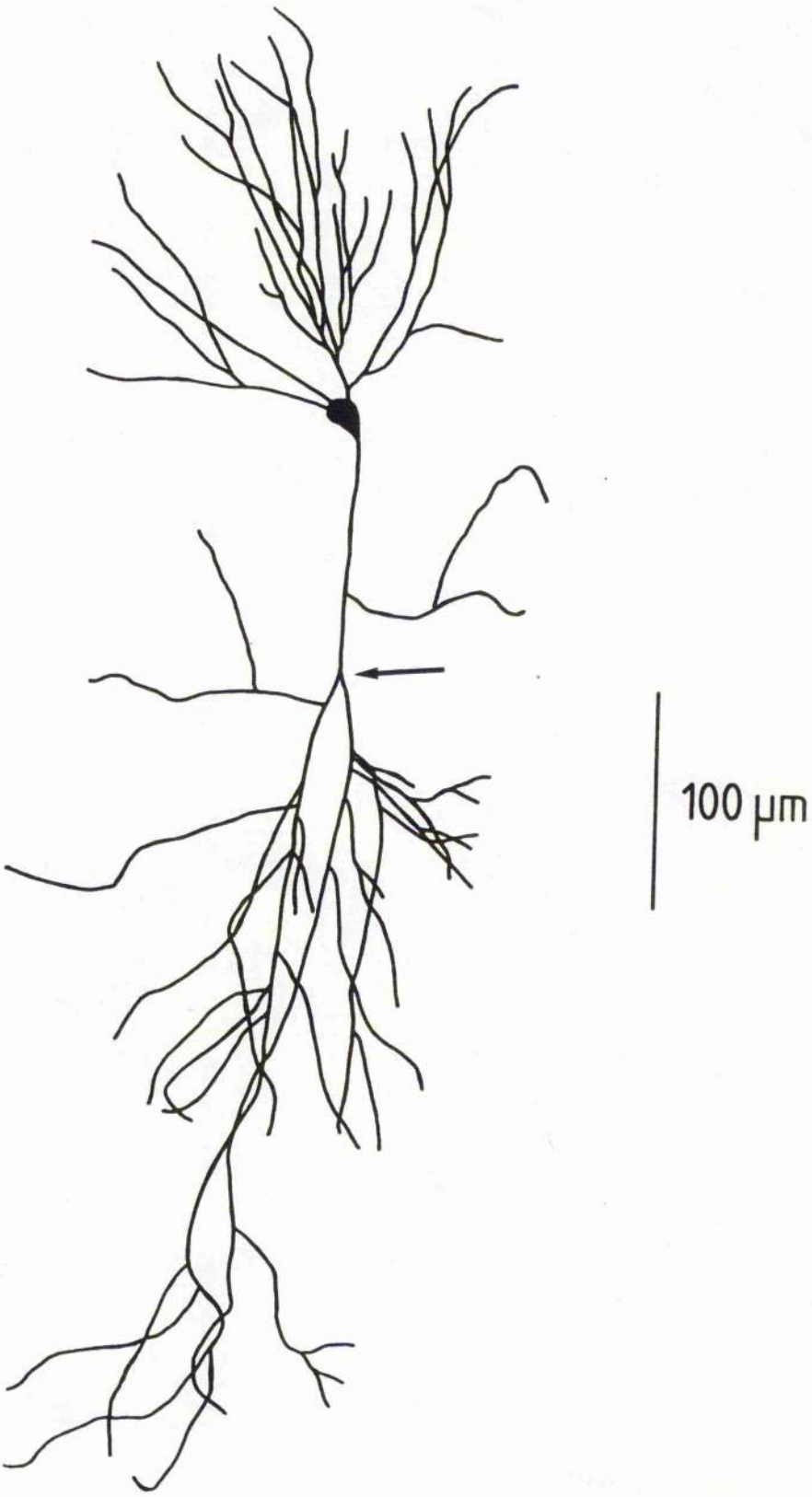
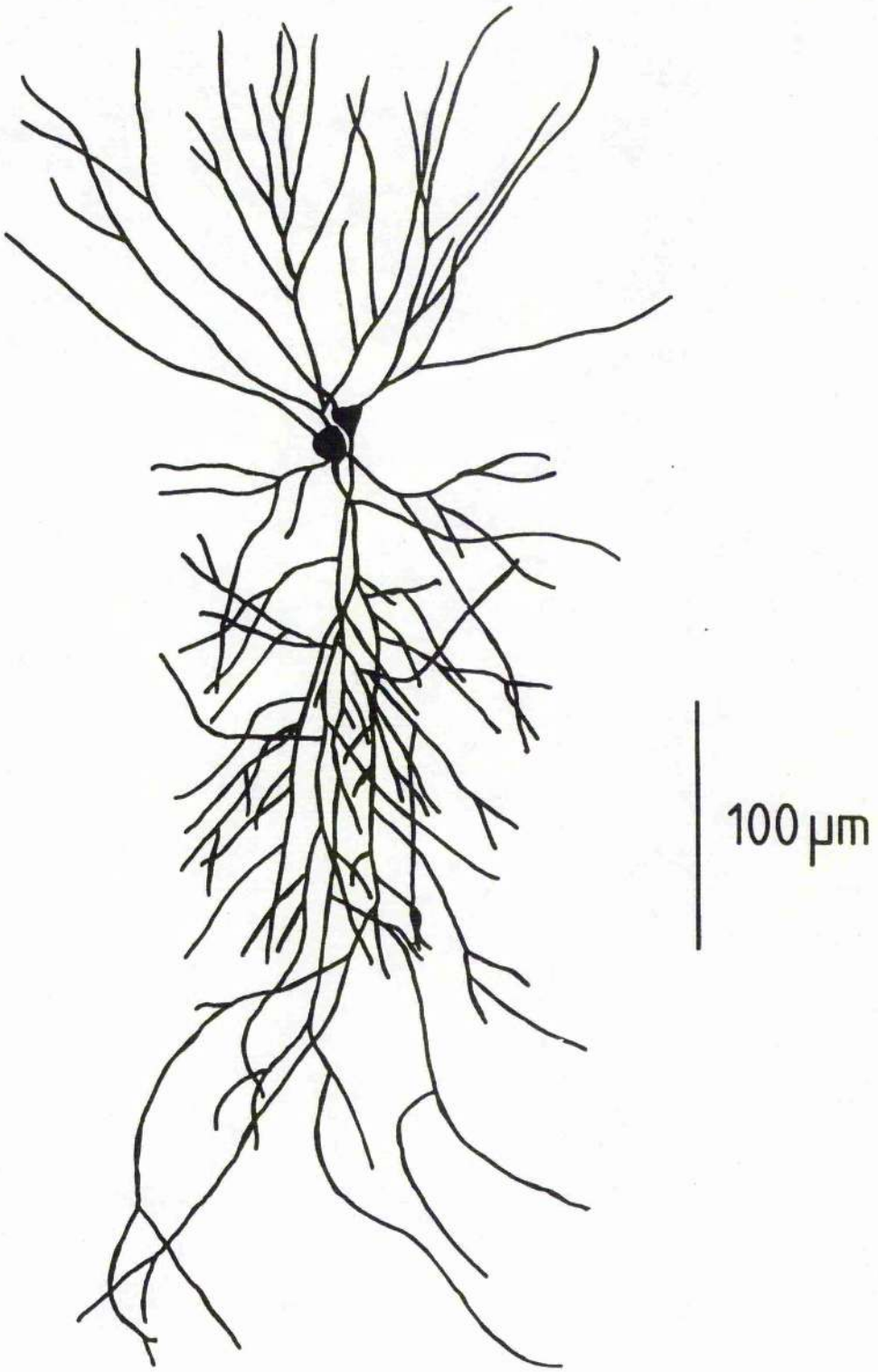

100 µm

Fig. 4.5 Reconstruction of two CA1 pyramidal neurones which were dye filled after injection of a single neurone. The evidence suggested that the dye moved directly from one neurone to the next.


100 µm

Within the basal dendritic field, the axon of an injected neurone could not be distinguished from the basal dendrites. The axon could only be identified if it was traced out of the basal dendritic field: this was possible on seven occasions. The axon was observed to project in the alveus, caudally, toward the subiculum. In one case, in addition to the axon projecting caudally, a collateral was observed to project rostrally in the alveus toward the fimbria. Axons were traced for up to 500μm and then the axon then passed out of the slice or the intensity of fluorescence in the axon decreased so that it could be traced no further. When the axon passed out of the slice, the point of exit was more intensely fluorescent than other parts of the process. When the axon was identified, it was observed to branch within the the dendritic field and that these branches terminated within the dendritic field.

On eight occasions, two neurones were observed to be dye filled after injection of a single neurone (Fig. 4.5). Two types of evidence suggested that the dye passed directly from one neurone to the next. First, on microscopical examination of the slices, dye was not seen in the extracellular space. Second, during the injection, there were no signs from the potential recordings that the electrode had moved from one neurone to the next.

4.3 DYE INJECTIONS IN THE CA3 REGION

Fifteen neurones were injected with Lucifer Yellow CH in the CA3 region: thirteen single neurones and four double filled

Fig. 4.6 Photographic montage of a CA3 pyramidal neurone injected with Lucifer Yellow. Note that since the dendrites pass into and out of the focal plane, some dendrites may not be shown and some may not appear complete. However, the reconstruction of this neurone (Fig. 4.7) shows all the dendritic processes of this neurone which were observed.

The marker bar represents 75μm.

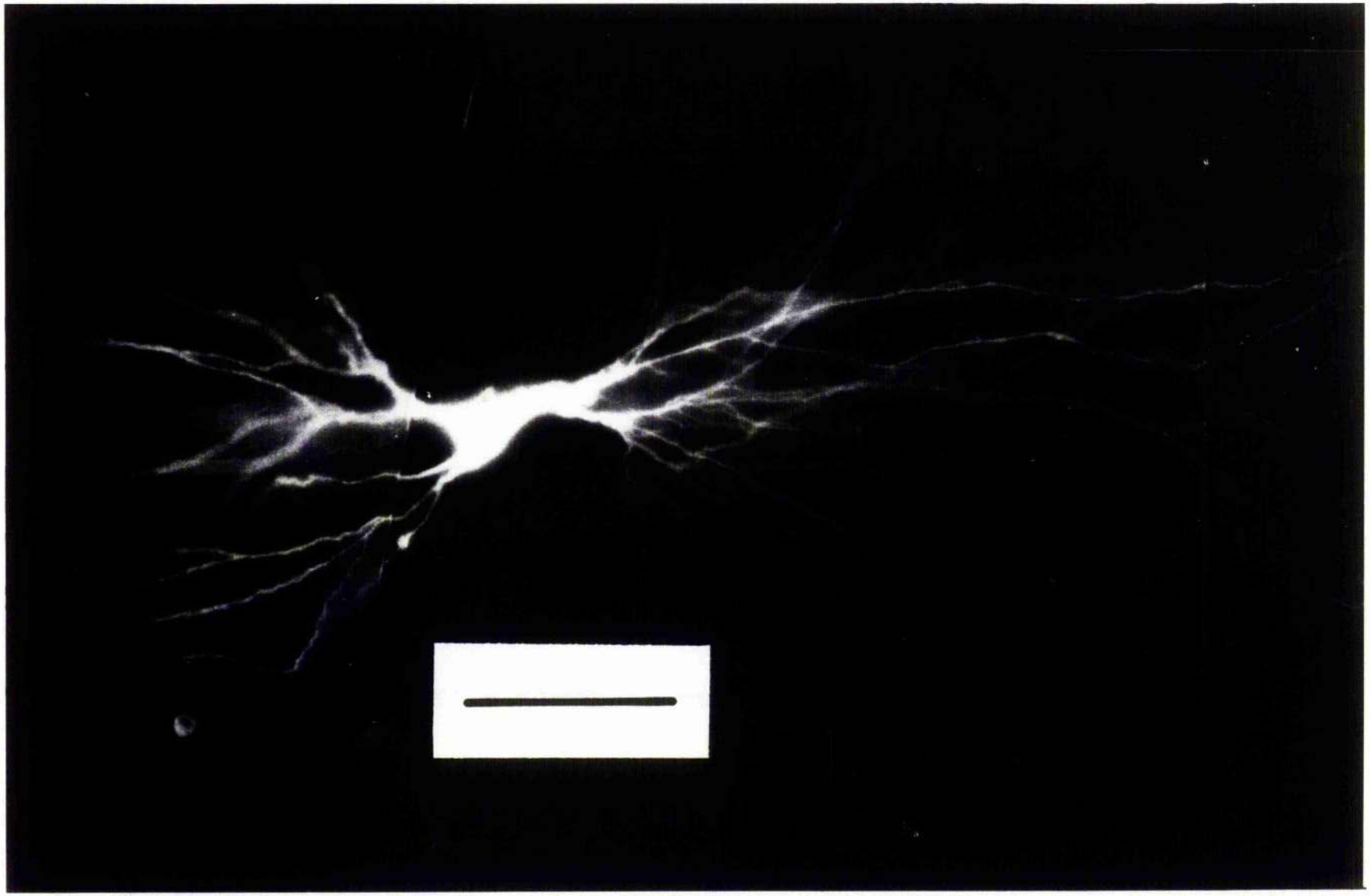
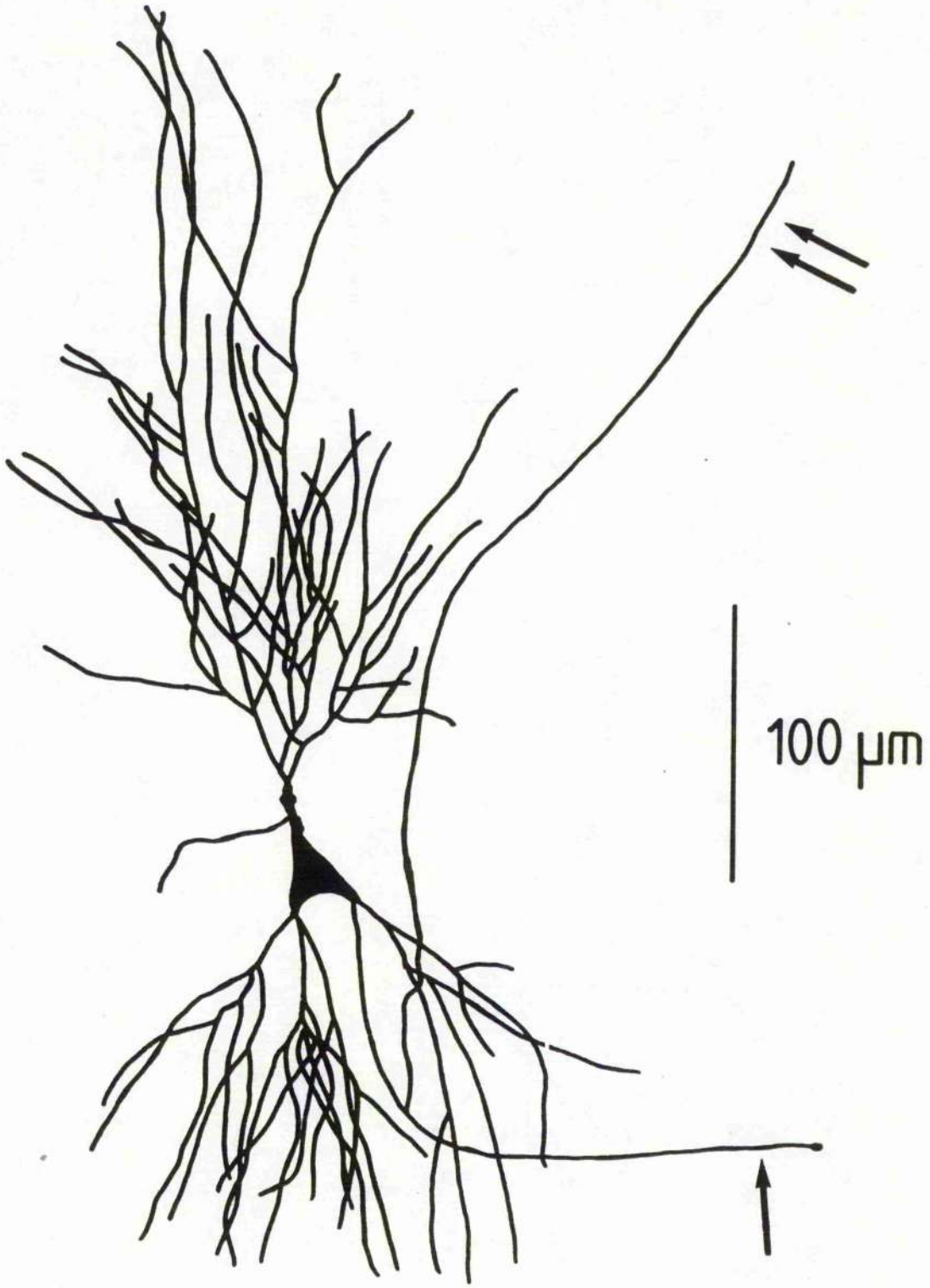


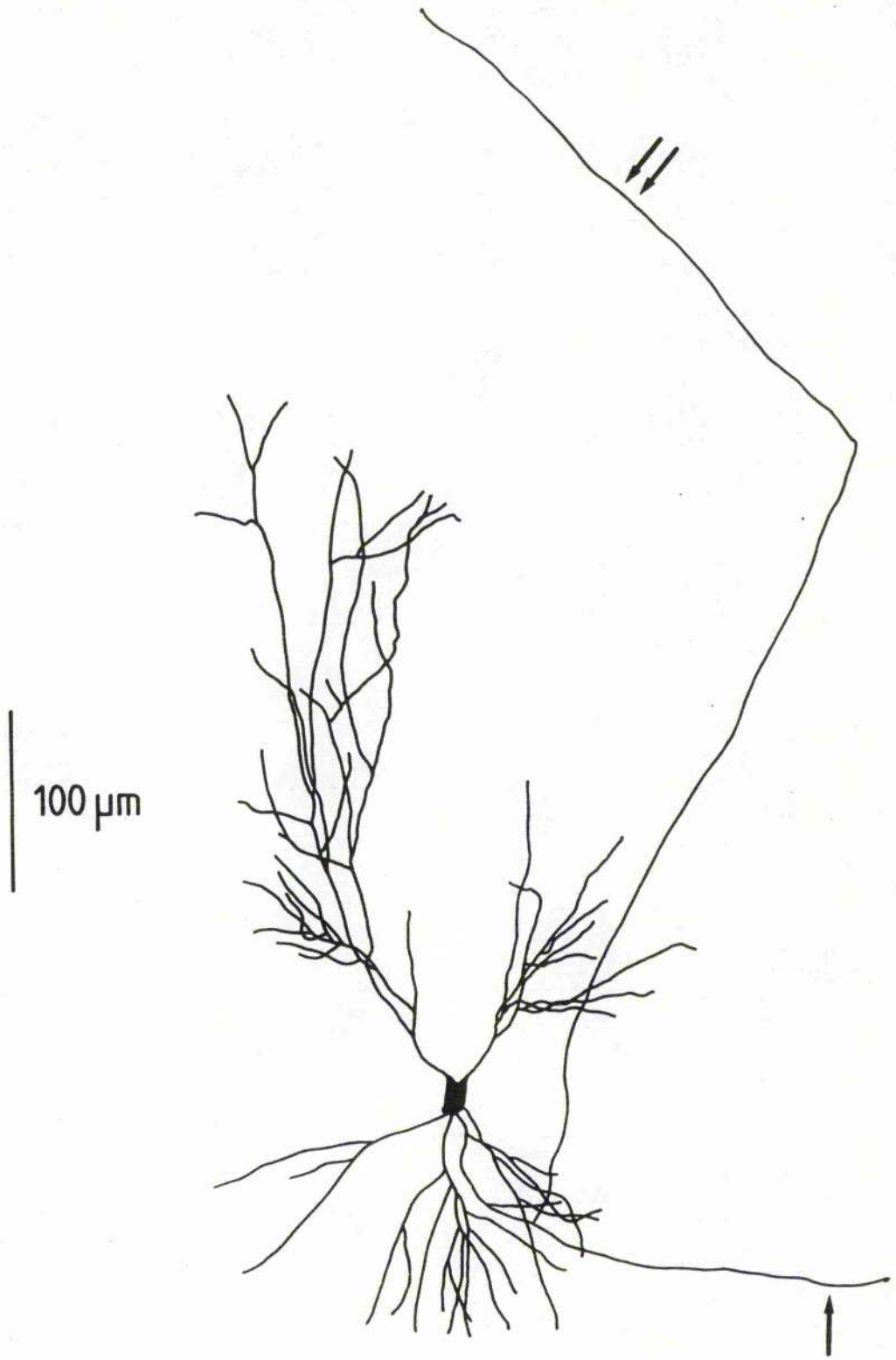
Fig. 4.7 Reconstruction of the CA3 pyramidal neurone shown in Fig. 4.6. The axon (single arrow) was identified and traced towards the fimbria and the Schaffer collateral was traced through the s. pyramidale and into the apical dendritic layers.


100 µm

neurones (two injections) were examined and reconstructed in two dimensions (Figs. 4.6 & 4.7). The general morphology of the injected neurones was consistent with descriptions of pyramidal neurones in this region (Cajal, 1911; Lorente de No, 1934; Harris, Cruce, Greenough, & Teyler, 1980). However, the structure of these neurones differed from the pyramidal neurones injected in the CA1 region in certain respects. The perikarya were 20µm to 30µm in diameter measured perpendicular to the dendritic axis. Several branching dendrites were observed to project from the basal surface of the perikarya. These dendrites were similar to the basal dendrites of CA1 neurones and were traced for about 150um to the alveus. Usually more than one dendrite projected from the apical surface of the perikarya and these could be traced ss far as the fissure (about 350µm). The proximal part of the dendrites was thick and beaded compared to the fine, smooth dendrites of the CA1 neurones. However, similarly to injected CA1 neurones, clearly defined spines on either the apical or the basal dendrites were not observed.

The axon from each of 14 pyramidal neurones in this region was identified. The axons were traced through the basal dendritic field for up to 300µm toward the fimbria. When the axon was observed to project out of a slice, the point of exit was observed to be more fluorescent than other parts. The axon of the CA3 pyramidal neurone branched within the basal dendritic field in a similar way to the axon of CA1 neurones. These branches did not project out of the dendritic field. In some cases, the axon was observed to divide at the edge of the basal dendritic field to give

Fig. 4.8 Reconstruction of a CA3 pyramidal neurone injected with Lucifer Yellow. This was the only case in which the Schaffer collateral (double arrow) was traced through the apical dendrites of the CA3 region, around the end of the fissure, and into the apical dendrites of the CA1 pyramidal neurones. The axon (single arrow) was also identified.


100 µm

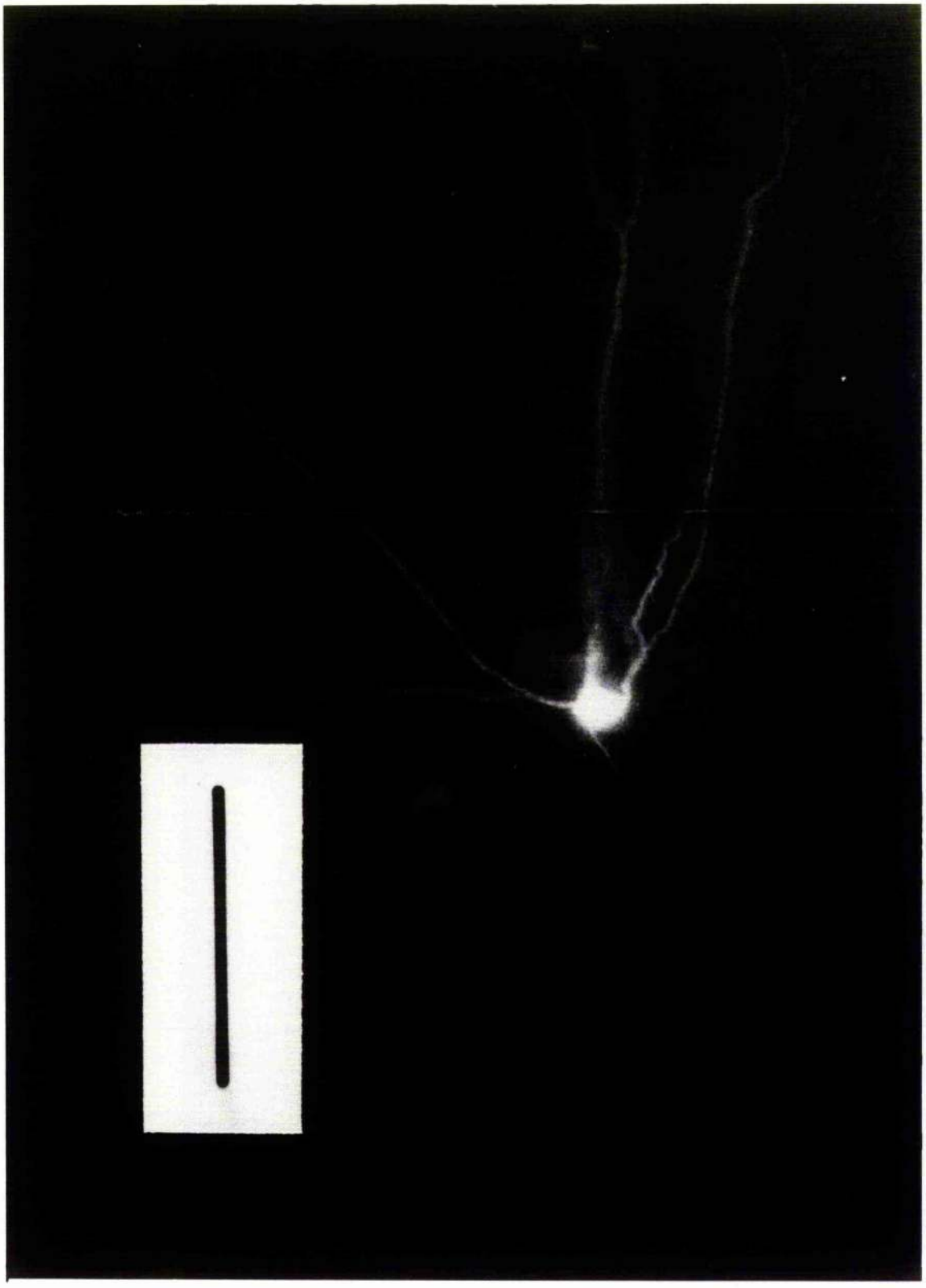
off a collateral. The collateral was identified, from its projection through the stratum pyramidale and into the stratum radiatum and stratum lacunosum of the CA3 region, as the Schaffer collateral (see Fig. 4.7). In one case, the collateral was observed to pass through the apical dendritic field of the injected neurone, around the end of the fissure and into the apical dendrite field of CA1 region at approximately the level of the stratum lacunosum (Fig. 4.8).

When two neurones were filled with dye after a single injection, there was no evidence of dye in the extracellular space. Nor was there any indication that the electrode had moved from one neurone to the next during dye injection. The dye apparently moved directly from one neurone to the next.

4.4 DYE INJECTIONS IN THE AREA DENTATA

It was not as easy to make good penetrations of neurones in the area dentata as in the CA1 or CA3 regions. Consequently injections of Lucifer Yellow CH into granule cells were less frequently made compared to injections into pyramidal neurones. Eight satisfactory injections of dye into granule cells were made, but in only two cases was a single neurone later observed to be dye filled (Figs. 4.9 & 4.10). On the other occasions, more than one neurone was found to be filled with dye (Fig. 4.11). All granule cells injected with Lucifer Yellow were in the part of the area dentata opposed to the hippocampal fissure.

Fig. 4.9 Photographic montage of a single granule cell of the area dentata injected with Lucifer Yellow From the perikaryon, the dendrites projected into the molecular layer towards the fissure. The axon projected in the opposite direction. The marker bar represents 75µm.



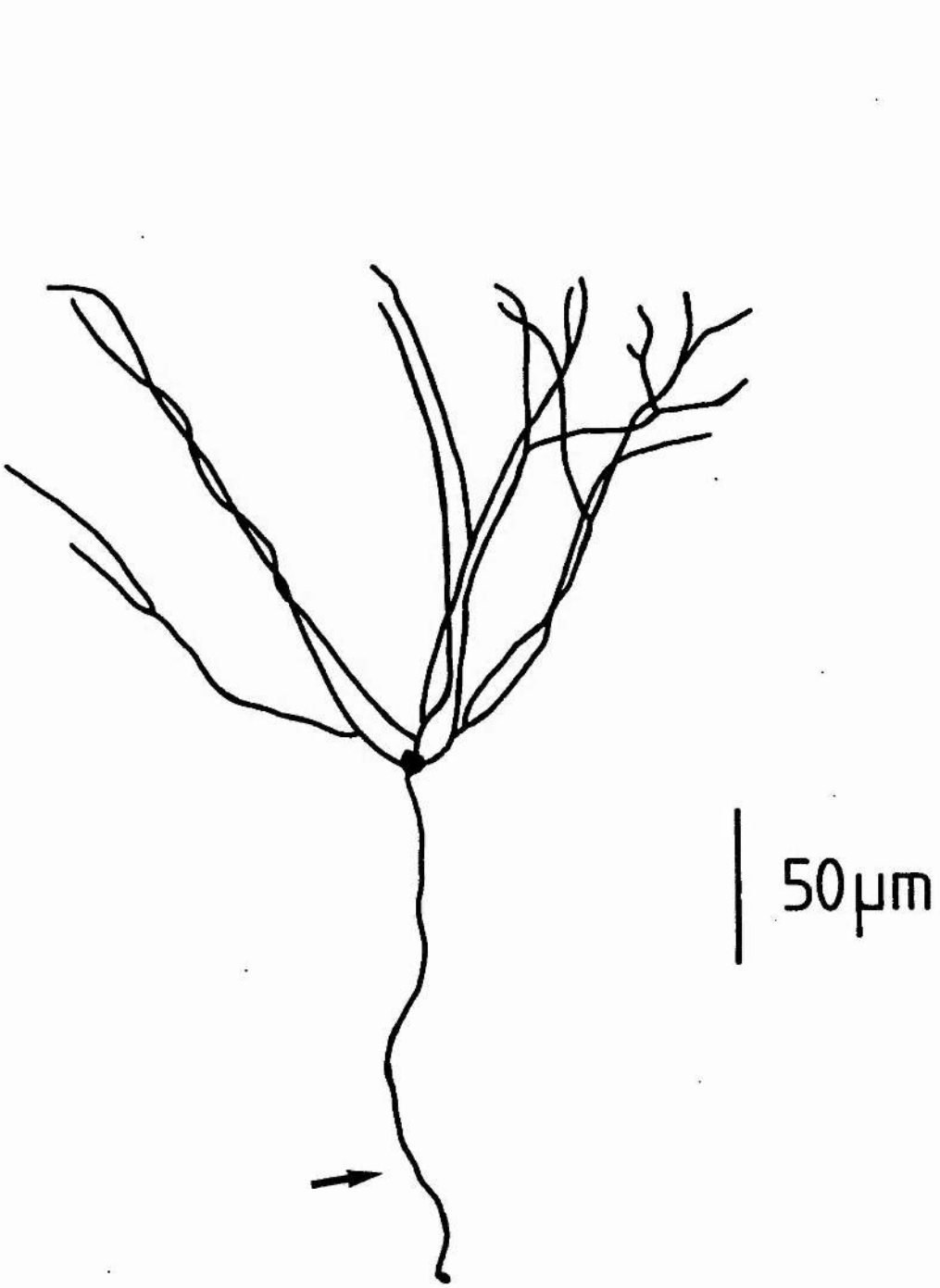

50µm

Fig. 4.10 Reconstruction of the same granule cell shown in Fig. 4.9, with the axon marked (arrow).

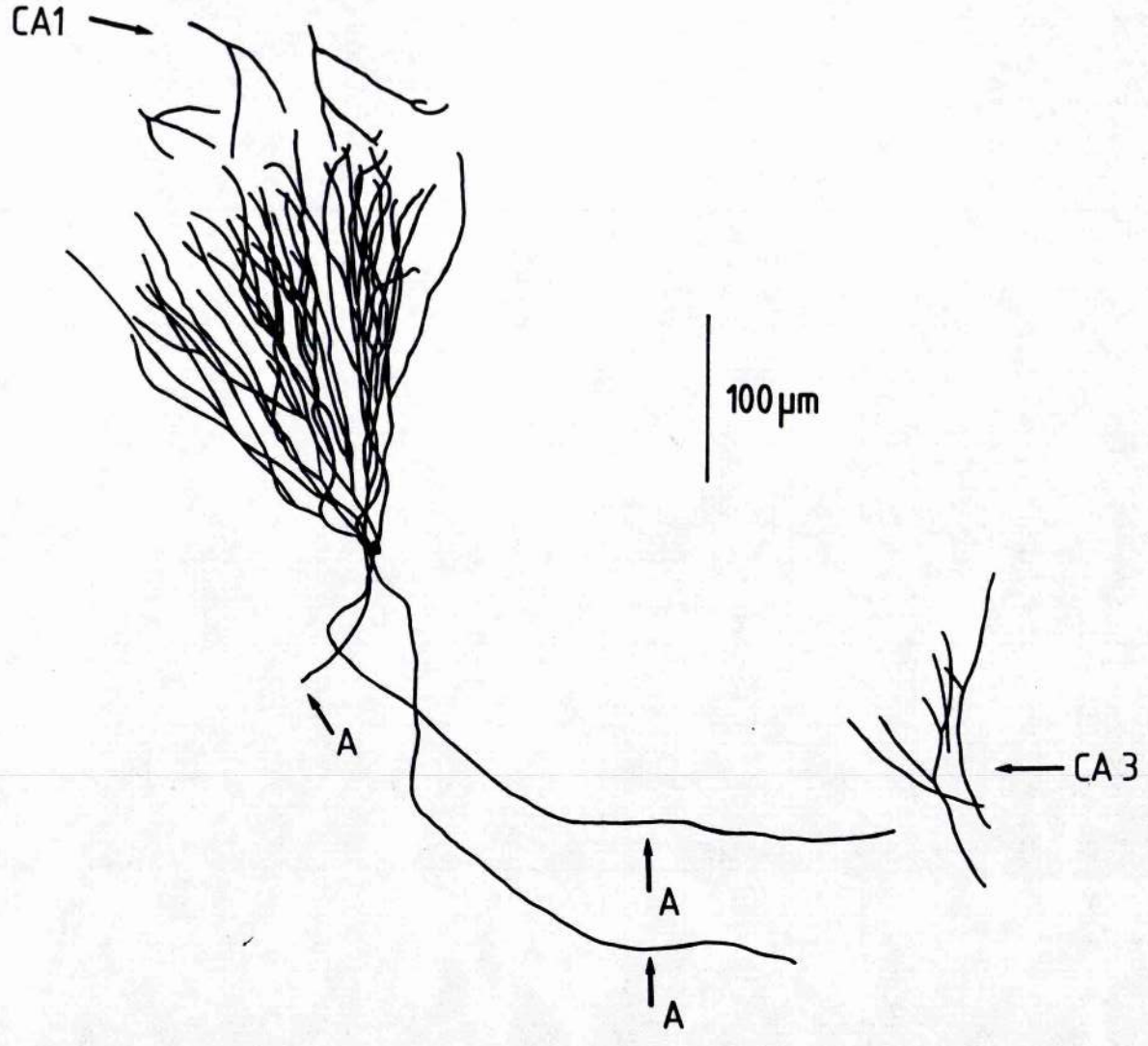

CA1
100 µm
A
A
A
CA 3

Fig. 4.11 Reconstruction of three granule cells filled with dye after injection of a single neurone. From the same slice, some of the distal apical dendrites of an injected CA1 pyramidal neurone (CA1), and some of the apical dendrites of an injected CA3 pyramidal neurone (CA3) are also shown. The dendrites of the granule cells are separated from those of the CA1 neurone by the fissure. The axons of two of the granule cells (A) were traced into the apical dendrites of the CA3 neurone.

The diameter of the perikarya of the granule cells was about 10μm. Dendrites projected from the perikarya in a fan shaped manner into the stratum moleculare as far as the fissure. The axon of each granule cell projected from the perikaryon in the opposite direction to the dendrites, and could be traced into the apical dendritic field of the CA3 pyramidal neurones (see Fig. 4.11). As with the injected pyramidal neurones, when more than one granule cell was filled with dye after injection of a single cell, it appeared that the dye had moved directly between the two cells.

CHAPTER FIVE

EFFECTS OF SEROTONIN, ITS PUTATIVE ANTAGONISTS, AND OTHER AGONISTS ON CA1 NEURONES

5.1 GENERAL OBSERVATIONS

Serotonin, acetylcholine or glutamic acid were applied from a micropipette positioned in the slice independently of the recording electrode. At first, the iontophoretic electrode was positioned in the slice before a neurone was satisfactorily impaled. However, it became routine to position the iontophoretic electrode after the satisfactory penetration of a neurone. The second method allowed more accurate positioning of the iontophoretic electrode, but during positioning of the electrode, the impalement could easily be disrupted.

In some experiments, the iontophoretic electrode was advanced into the slice without application of a retaining current. A "leakage" response was recorded from some neurones as the electrode was advanced. These responses were the same as those produced by iontophoresis. When leakage responses were recorded, the iontophoretic electrode could be positioned more accurately.

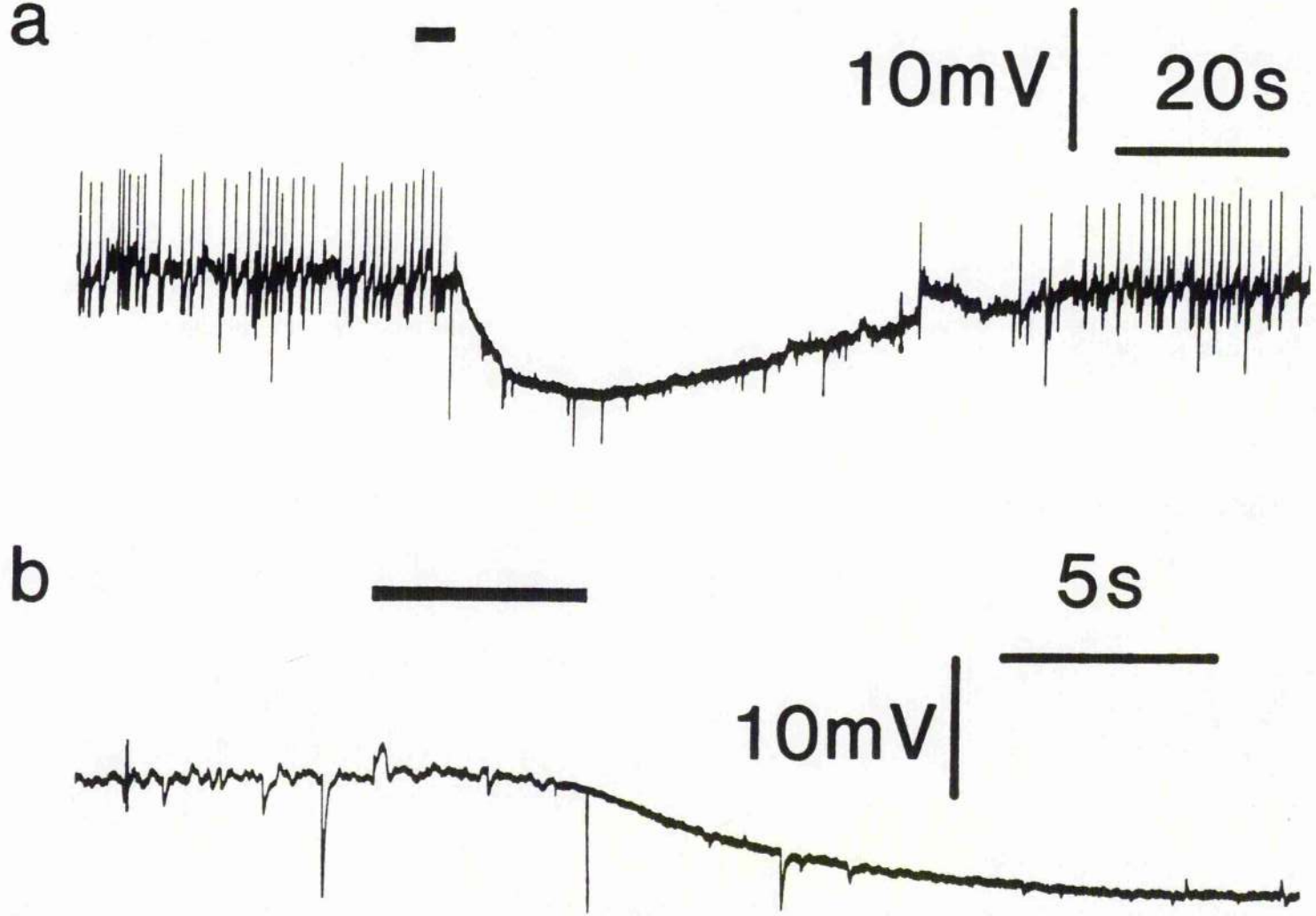
Many neurones did not respond to an applied agonist: potential changes, and changes of the spontaneous spike frequency during or following the iontophoretic current ejection pulse were not observed. The lack of response was not necessarily due to insensitivity of the neurones to the agonist. Rather, desensitisation or incorrect positioning of the iontophoretic electrode were more probable in these cases. This problem prevented proper investigation of the sensitivity of the different layers of the hippocampus to iontophoresed agonists.

Fig. 5.1; Records to show the effect of iontophoresed serotonin on a CA1 pyramidal neurone at the resting potential (-63mV). In each of the records, made 10min apart and at different recorder speeds, serotonin was iontophoresed for the period shown by the bar above the potential trace.

a: This record shows that serotonin produced a membrane hyperpolarisation and reduced the frequency of spontaneous firing. The response lasted about 55s.

b: This record shows that the serotonin induced hyperpolarisation was not apparently associated with an increased frequency of i.p.s.p.'s and that there was a delay of over 2s between the start of the iontophoretic current pulse and the onset of the hyperpolarisation.

In this and all other records shown in this Chapter, the amplitude of action potentials has been greatly reduced by the pen recorder.


a
10mV
20s
b
5s
10mV

5.2 THE SEROTONIN RESPONSE

Serotonin was iontophoresed onto the apical dendrites within 200um of the stratum pyramidale. Responses to serotonin were rarely recorded when the iontophoretic electrode was positioned in the more distal apical dendrites, or in the basal dendritic field.

5.2A Effect of Serotonin on Resting Potentials.

All neurones, which were sensitive to serotonin, responded in the same way independent of the resting potential. Serotonin produced a membrane hyperpolarisation of between 1mV and 10mV (Fig. 5.1). The onset of the response was between 2s and 10s after the start of the iontophoretic ejection pulse. The response latency period was dependent on the position of the iontophoretic electrode: movement of the electrode produced an increased or decreased latency. The latency period was therefore in part equivalent to the diffusion of serotonin from the electrode tip to the receptors on the neurone. The maximum hyperpolarisation occurred between 5s and 20s after the termination of the iontophoretic current ejection pulse. The response duration was between 30s and 3min.

The membrane hyperpolarisation was not due to the ejection current pulse itself. Although current artefact was often recorded, it had the same form regardless of the subsequent potential change. Artefacts were observed: 1) without a subsequent membrane hyperpolarisation, 2) superimposed upon the

hyperpolarisation, and 3) preceding the onset of the hyperpolarisation by up to 10s.

A major part of the study was intended to be concerned with the effect of changes of the ionic composition of the ACSF and of putative serotonin antagonists on the response. A minimum response amplitude of -2.5mV at the resting potential was therefore introduced as the criterion for a satisfactory response. This criterion was in addition to those defining a satisfactory impalement, and was only satisfied in about 10% of neurones tested. The criterion was more frequently satisfied when the normal K^+ concentration in ACSF was 3.75mM.

5.2B Effect of Serotonin on the Spike Frequency.

Accompanying the hyperpolarisation produced by serotonin, there was a decreased frequency of spontaneous action potentials. This decrease followed the same time course as the hyperpolarisation. A rebound excitation was sometimes observed: during the later part of the recovery of the resting potential and for up to 30s afterwards, the spike rate was greater than normal.

The spontaneous spike frequency was more noticeably affected when the normal K^+ concentration in ACSF was 6.25mM. Serotonin produced a dramatic reduction of the spike frequency, but the membrane hyperpolarisation was small or not observed at all. In contrast, when the normal K^+ concentration of ACSF was 3.75mM, the resting spike frequency was generally lower and the effect of

Fig. 5.2 A series of serotonin responses of a CA1 pyramidal neurone, each separated by 3min, showing that the response amplitude was dose dependent. In every case, serotonin was iontophoresed for 2s, as shown by the bar above each potential trace. The intensity of the current pulse in each case was: a:- 30nA, b:- 50nA. c:- 70nA, d:- 80nA, and e:- 90nA.

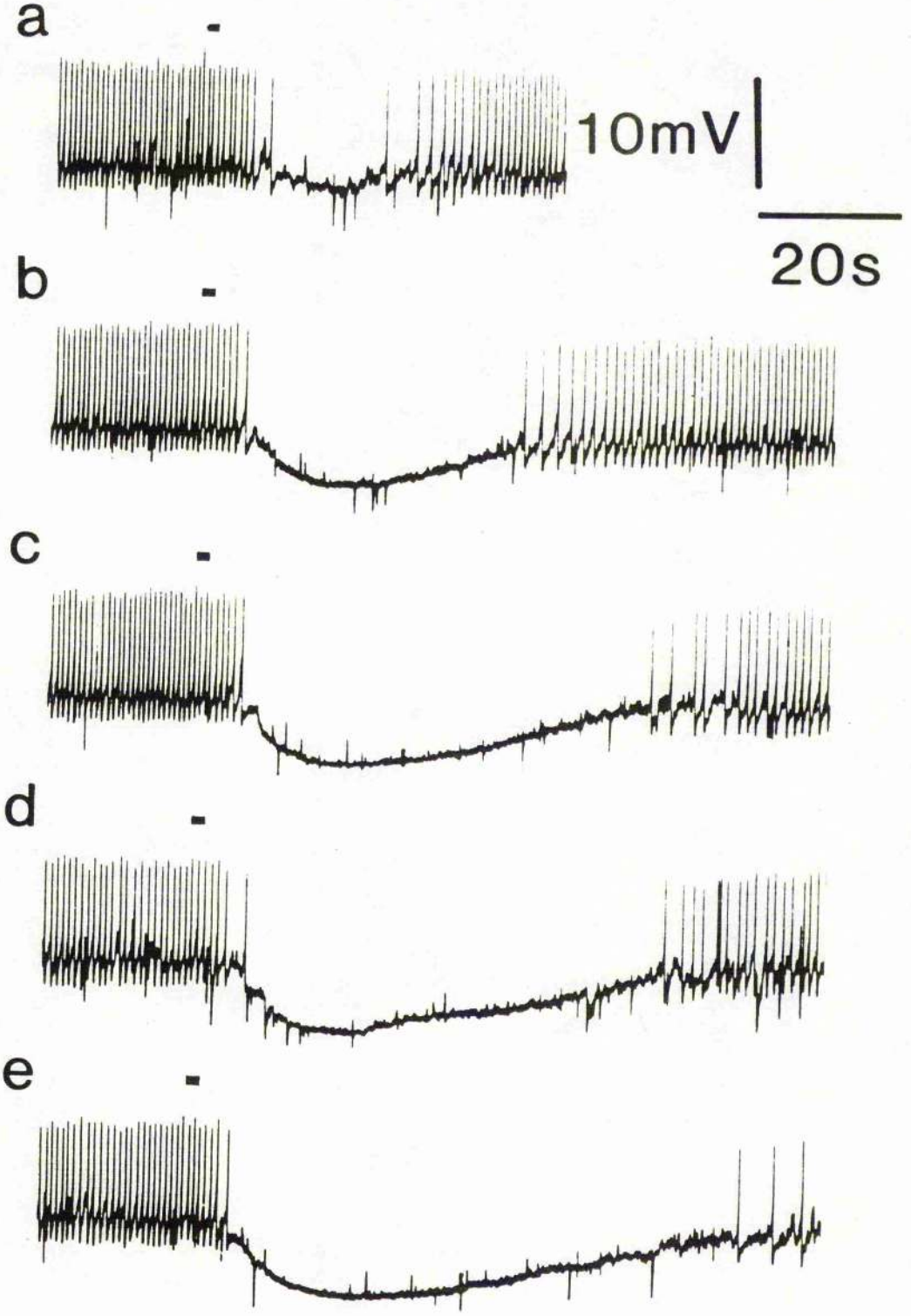
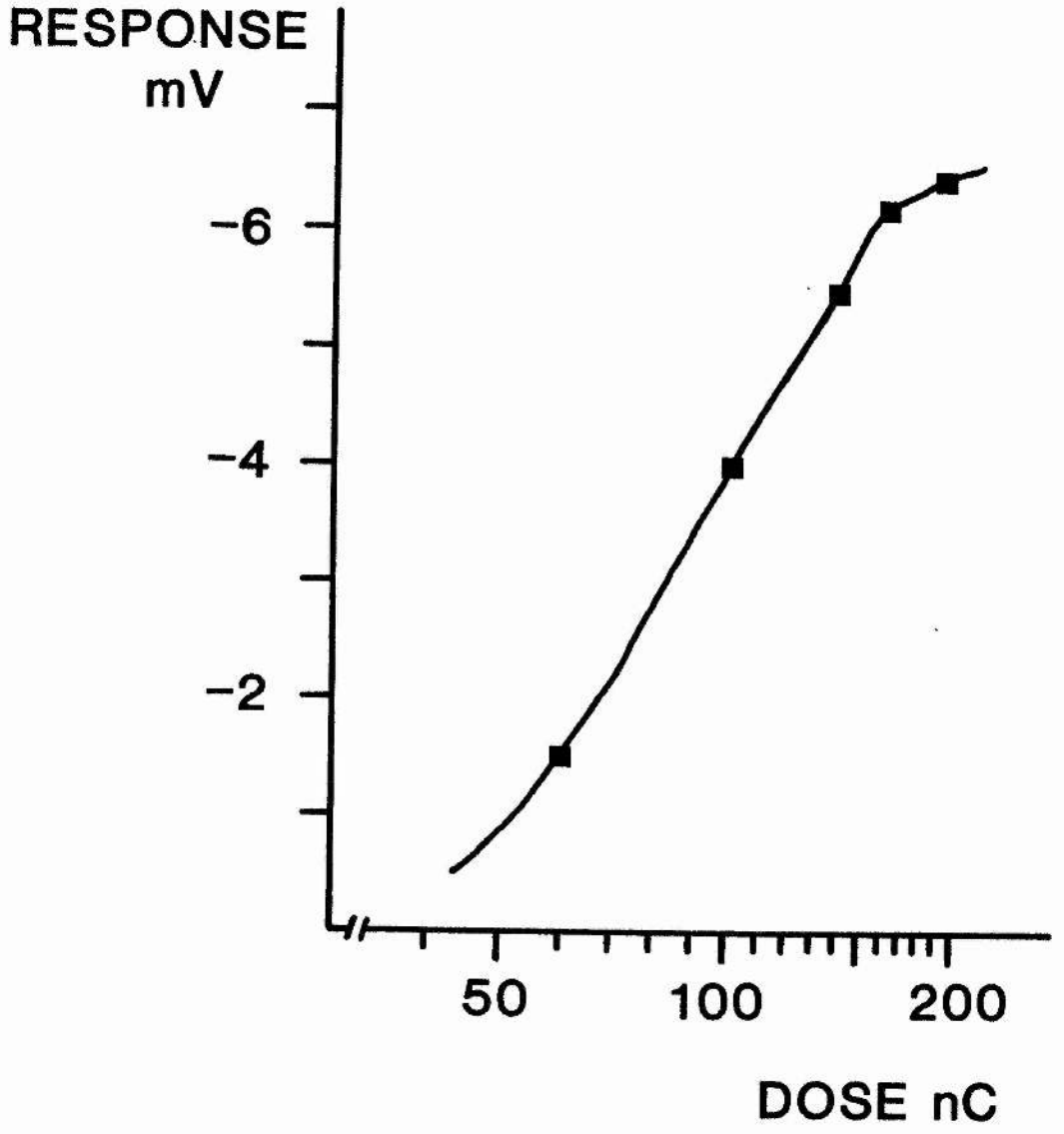

a
b
c
d
e
10mV
20s

Fig. 5.3 The relationship between the dose of iontophoresed serotonin and the response amplitude obtained from the records shown in Fig. 5.2, and plotted semi-logarithimically. At high doses, the response appears to be reaching a maximum, which would be expected 1) if the membrane hyperpolarised as far as the reversal potential of the response, and/or 2) if the serotonin response was receptor mediated.


RESPONSE
mV
-6
-4
-2
50
100
200
DOSE nC

serotonin on the spike frequency was less dramatic. The effect of serotonin on the spontaneous spike frequency was not analysed quantitatively.

5.2C Effect of Serotonin on Spontaneous Synaptic Potentials.

The frequency of spontaneous i.p.s.p.'s was not changed during any part of the serotonin induced hyperpolarisation. If the effects of serotonin on the pyramidal neurones were mediated indirectly via an inhibitory interneurone, the frequency of spontaneous inhibitory synaptic potentials might be expected to increase following serotonin application. A direct effect of serotonin was therefore indicated.

5.2D Dose-Dependency of the Response.

The "dose" (nC) of serotonin applied during a single iontophoretic current ejection pulse was defined as the product of the duration (s) and the intensity (nA) of the pulse.

The response amplitude was dependent on the dose of serotonin (Fig. 5.2). Alteration of the duration or the intensity of the current pulse produced a similar change of the response amplitude. The relationship between dose and response amplitude is shown in Fig. 5.3, and is typical of a receptor mediated response.

Repetition of the same dose of serotonin at intervals of between 2min and 6min, produced consecutive responses of

approximately constant amplitude. The interval between consecutive doses was set so that each dose was applied at least 30s after complete recovery of the potential change produced by the previous dose. Although consecutive responses were almost the same, there was often a gradual decline of the response amplitude over a period of an hour or more. This change represented the observed progressive block of the iontophoretic electrode.

The response duration was also dose dependent: with increasing doses of serotonin, the duration increased. The duration of the response to consecutive applications of the same dose of serotonin was more variable than the response amplitude. Consequently, the response duration, and changes of the duration produced by alteration of the composition of ACSF, were not analysed quantitatively.

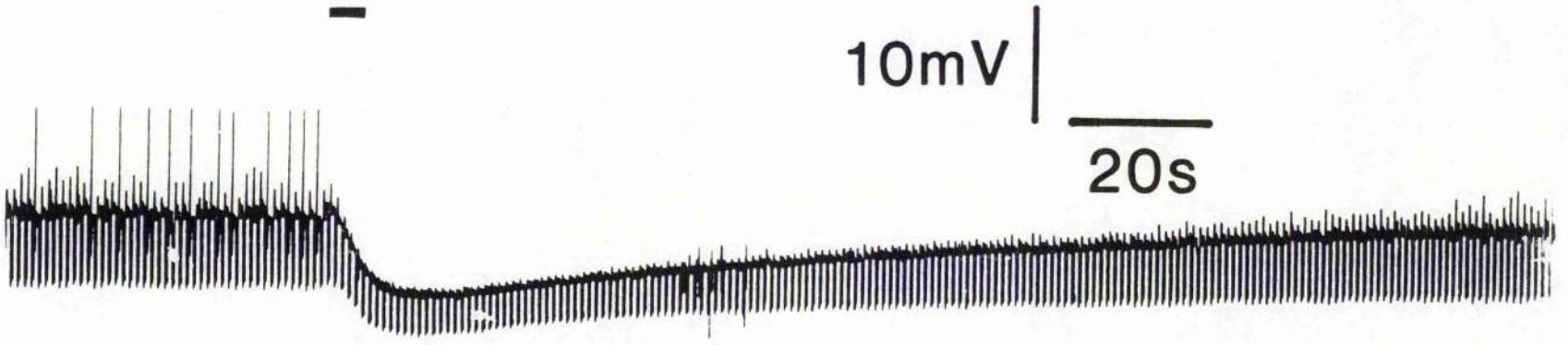
5.3 IONIC BASIS OF THE SEROTONIN RESPONSE

The events underlying the membrane hyperpolarisation and spike frequency decrease were examined to determine the ionic basis of the serotonin response. The effect of serotonin on the input membrane resistance, the effects of membrane potential on the serotonin response, and the effects of changes of the ionic composition of ACSF on the response were therefore investigated.

5.3A Effects of Serotonin on the Input Membrane Resistance.

Injected constant current hyperpolarising pulses (1Hz, 350ms)

Fig. 5.4 This record shows the effect of serotonin on the input resistance of a CA1 pyramidal neurone. Constant current hyperpolarising pulses were intracellularly injected and the resulting potential changes were recorded. Iontophoresis of serotonin, shown by the bar above the potential record, reduced the input resistance by 50% in addition to hyperpolarising the neurone.


10mV
20s

produced voltage deflections which were proportional to the input membrane resistance. Changes of the amplitude of these deflections therefore represented a similar change of the input membrane resistance.

Serotonin reduced the input resistance by up to 75% (Fig. 5.4). The onset and the peak of the resistance change followed the same time course as the potential change produced by serotonin. The recovery of the normal resistance lagged slightly behind the recovery of the potential. The serotonin induced reduction of the input resistance was also observed in those experiments where depolarising constant current pulses were injected rather than hyperpolarising pulses.

5.3B Effect of Membrane Potential on the Response.

Membrane hyperpolarisation with a decreased input membrane resistance was indicative that serotonin produced an increased membrane permeability to K^+ and/or Cl^- ions. To examine which ionic species was involved, the reversal potential of the response was determined. The effect of serotonin was first observed at the resting potential, and then at depolarised and hyperpolarised levels. These potentials were set by injection of steady currents. In addition, the input membrane resistance was also monitored throughout these experiments as described previously.

A frequently encountered problem in these experiments was the instability of the recordings during prolonged current injection.

Fig. 5.5 Records from an experiment on a CA1 pyramidal neurone to show the effect of the same dose of serotonin at different preset membrane potentials. In each part of the figure, iontophoresis of serotonin is marked by a bar above the potential trace, and to the right of the potential trace, the preset potential is given. The resting potential of the neurone was -76mV.

As the potential was increased, the hyperpolarisation produced by serotonin decreased and then the response reversed to become a depolarisation. Note that as the potential increased, the peak of the response was later than at lower potentials.

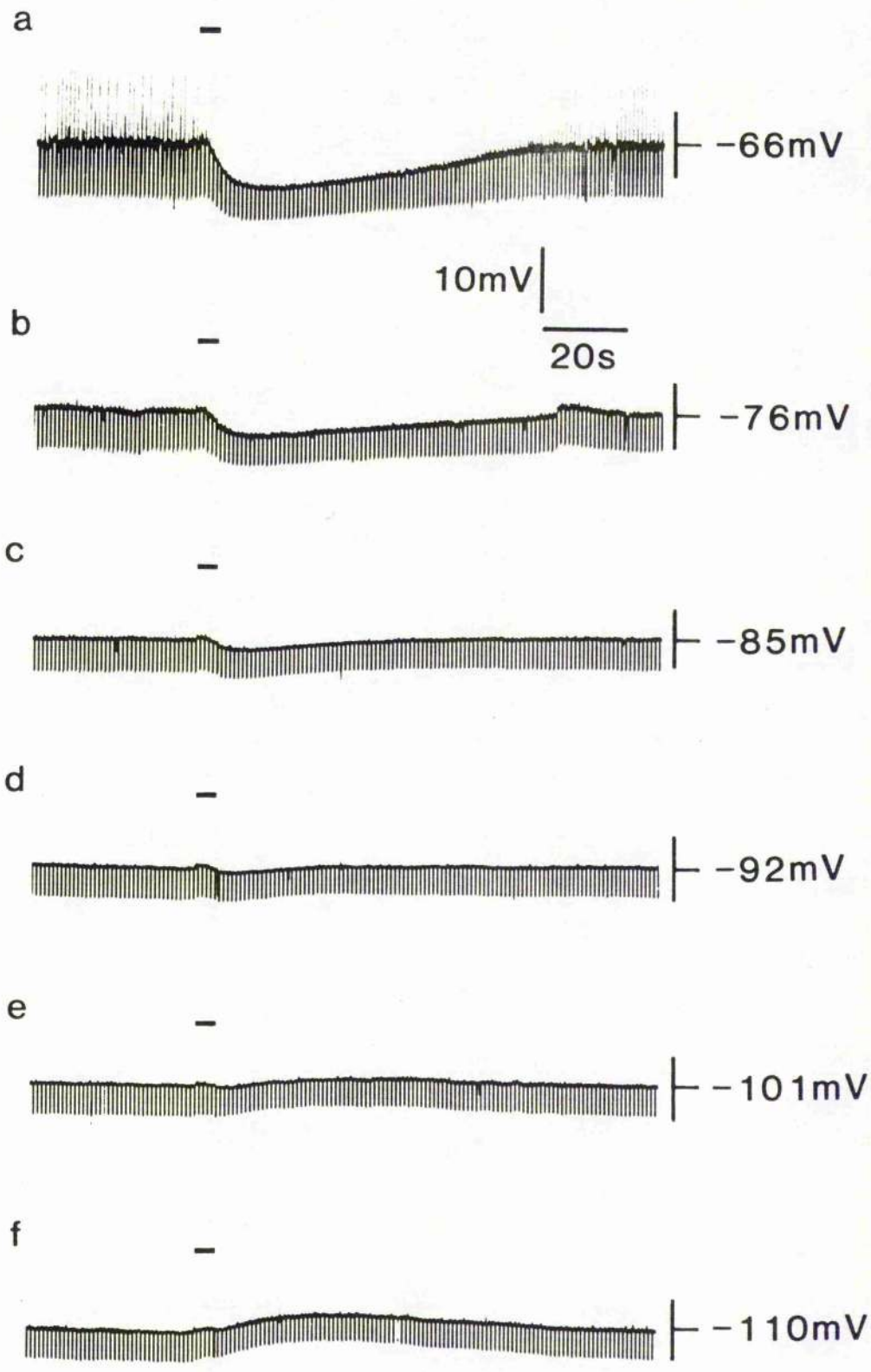
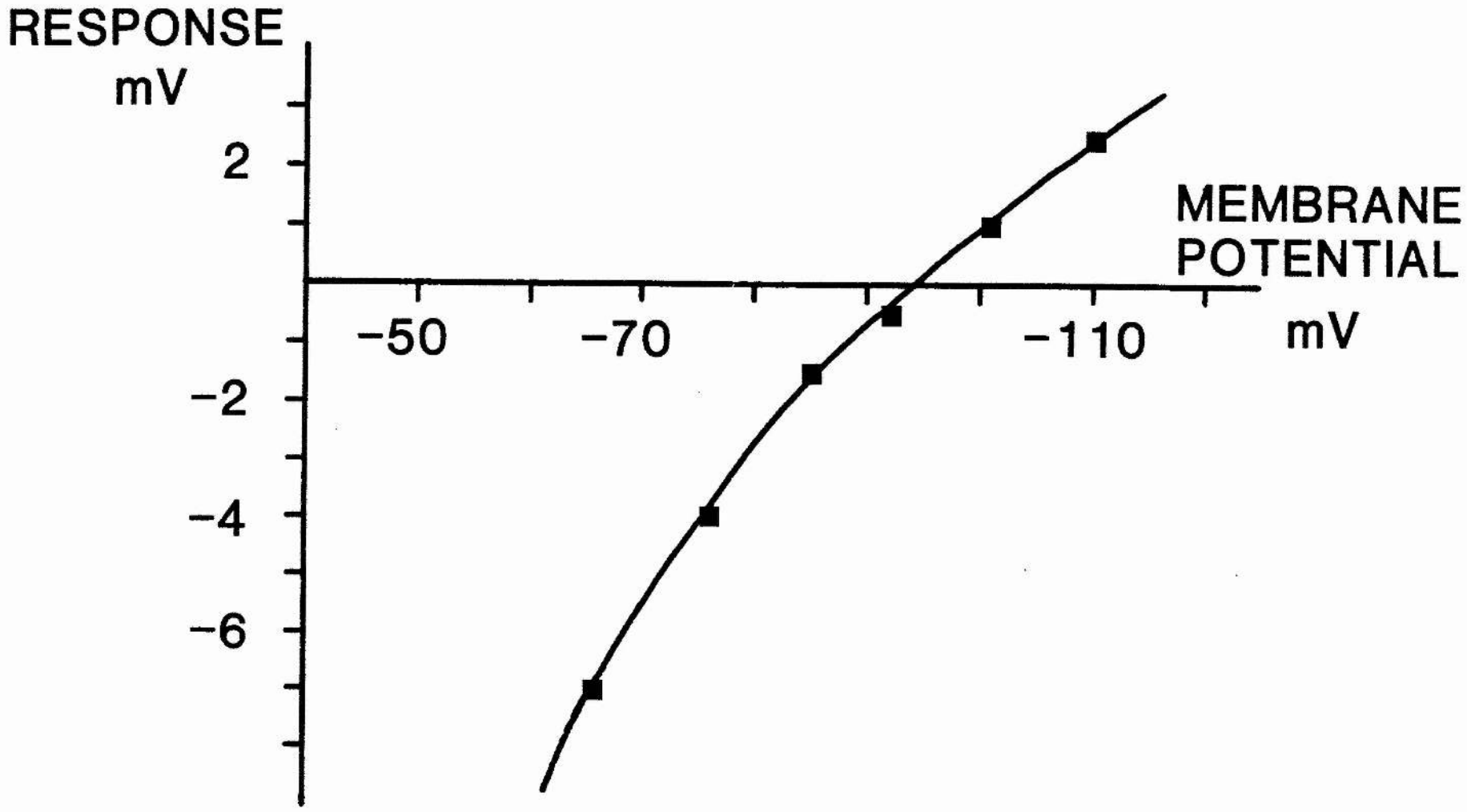

a
-66mV
10mV
b
20s
-76mV
c
-85mV
d
-92mV
e
-101mV
f
-110mV

Fig. 5.6 The relationship between the preset membrane potential and the peak amplitude of the serotonin response plotted from the data obtained from the records shown in Fig. 5.5. Note that the relationship is not linear.


RESPONSE
mV
2
−2
−4
−6
−50
−70
−110
MEMBRANE
POTENTIAL
mV

This instability was recorded as increased electrode noise or as either transient or more prolonged potential changes.

At depolarised levels, the serotonin induced hyperpolarisation was increased. As the potential was increased, the amplitude of the hyperpolarisation decreased and then reversed so that serotonin produced a membrane depolarisation (Fig. 5.5). The reversal potential of the response was between -84mV and -101mV, indicative that potassium was the ionic species involved in the response. However, the relationship between the response amplitude and the membrane potential was not linear (Fig. 5.6): at potentials greater than the reversal potential, the depolarising serotonin response was not as great as expected. In addition, the peak of the response appeared to be delayed at these high potentials.

5.3C Effects of Changes of the Ionic Composition of ACSF on the Response.

The hyperpolarisation produced by serotonin appeared to result from an increased membrane permeability to potassium ions. To confirm this indication, the effects on the serotonin response of changing the K^+ or Cl^- concentration of the ACSF, or of adding TEA to the ACSF, were therefore examined.

In these experiments, the serotonin response of a neurone was first established in normal ACSF. Then without interruption to the recordings, the composition of the ACSF entering the recording chamber was changed. After the effects of this change were

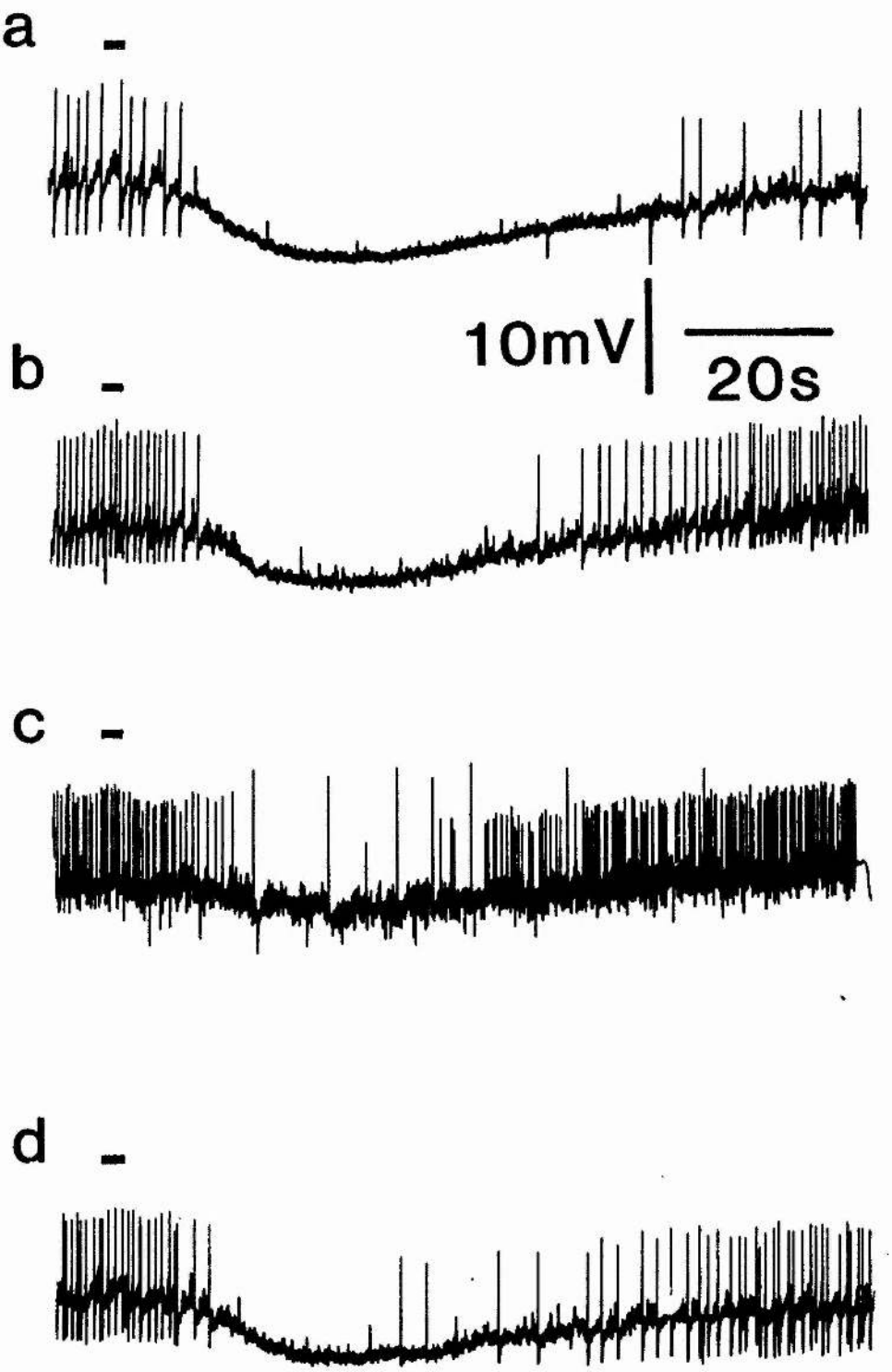
Fig. 5.7 Records to show the effect of the same dose of serotonin on a CA1 pyramidal neurone in different ionic conditions.

a: The response recorded while the slices were bathed in ACSF containing 3.75mM K^+.

b: The response recorded 6min after the K^+ concentration of ACSF introduced into the recording chamber was reduced increased to 13.75mM.

c: The response recorded 12min after the K^+ was increased to 13.75mM. The response amplitude has been greatly reduced, despite the 7mV depolarisation produced by the concentration change.

d: The response recorded a few minutes after the K^+ concentration in ACSF was returned to normal.


a
b
c
d
10mV
20s

recorded, normal ACSF was re-introduced into the recording chamber to "wash" the slices.

When the K^+ concentration was raised from 3.75mM to 13.75mM, the response amplitude was decreased by up to 75% (Fig. 5.7).. This decrease occurred gradually over a period of about 10min and despite the resting potential decrease produced by the increased K^+ concentration. When the normal ACSF was re-introduced into the chamber, the response amplitude returned to normal. In contrast, the response was not changed when the Cl^- concentration of the ACSF was reduced from 133mM to 72mM by substitution of half the NaCl with Na Isethionate.

A less dramatic decrease of the response amplitude was recorded in earlier experiments in which the K^+ concentration was raised from 6.25mM to 11.25mM. Conversely the response amplitude was slightly increased when the K^+ concentration was reduced from 6.25mM to 1.25mM.

Compared to the data obtained when the K^+ concentration of ACSF was changed, the effects of TEA (1mg/ml) on the response were less clear. In three experiments, the response amplitude was only slightly reduced by TEA although the effects of TEA on the spike duration and resting potential were observed. However in one experiment, the response was substantially reduced (65% reduction). In this experiment, the effect of TEA on the action potential duration and the resting potential were not greater than normal.

5.4 PHARMACOLOGY OF THE SEROTONIN RESPONSE

In this series of experiments, the effect of five supposed serotonin antagonists were examined. The original intention was to compare the concentration of each drug at which the response was reduced by 25%, 50%, and 75%. However this was not possible, and instead the concentration of a supposed antagonist which produced a substantial blockade of the response was determined.

In these experiments, the response of a neurone was established while the slices were bathed in normal ACSF. Then, without interruption of the recordings, ACSF containing a known concentration of a putative antagonist was introduced into the chamber for between 18min and 32min. The slices were then "washed" with normal ACSF. Since gradual spike frequency changes were normal and the initial spike frequency was less than 5Hz, the effects of the supposed antagonists on the spike rate were not systematically investigated.

Methysergide (100μM) produced a substantial blockade of the serotonin response within 15min of the introduction of methysergide-ACSF into the chamber (Fig. 5.8). After 20min, there was complete blockade of the response which was not reversible by up to 40min washing with normal ACSF. At lower concentrations (10μM and 50μM), the blockade was incomplete. In two experiments at these concentrations, the blockade appeared to be reversible in the first few minutes of washing with ACSF. After this period, a rapid block of the iontophoretic electrode occurred. Methysergide

Fig. 5.8 Records from an experiment showing that methysergide (100μM) reduced the serotonin response of a CA1 neurone to constant doses of serotonin (shown by the bar above the potential trace in each part of the figure).

a: The response while the slices were bathed in normal ACSF.

b: The response 8min after ACSF containing methysergide was introduced into the recording chamber.

c: The response 16min after ACSF containing methysergide was introduced into the recording chamber.

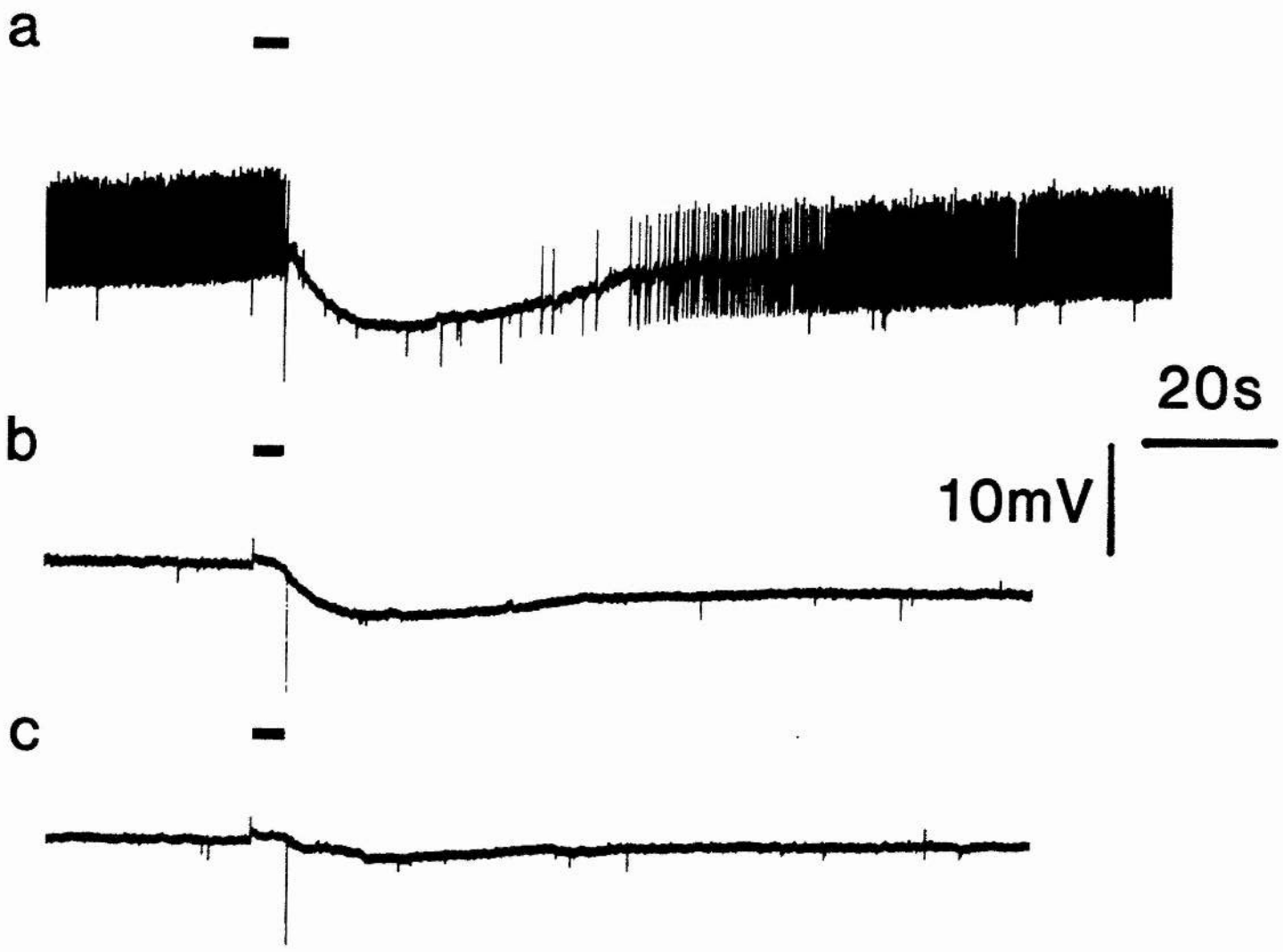

a
b
c
20s
10mV

Fig. 5.9 Records to show the failure of cyproheptadine (100μM) to block the response of a CA1 pyramidal neurone to constant doses of serotonin (shown by the bar above the potential trace in each part of the figure).
a: The serotonin response while the slices were bathed in normal ACSF.
b: The response 16min after ACSF containing cyproheptadine was introduced into the recording chamber.

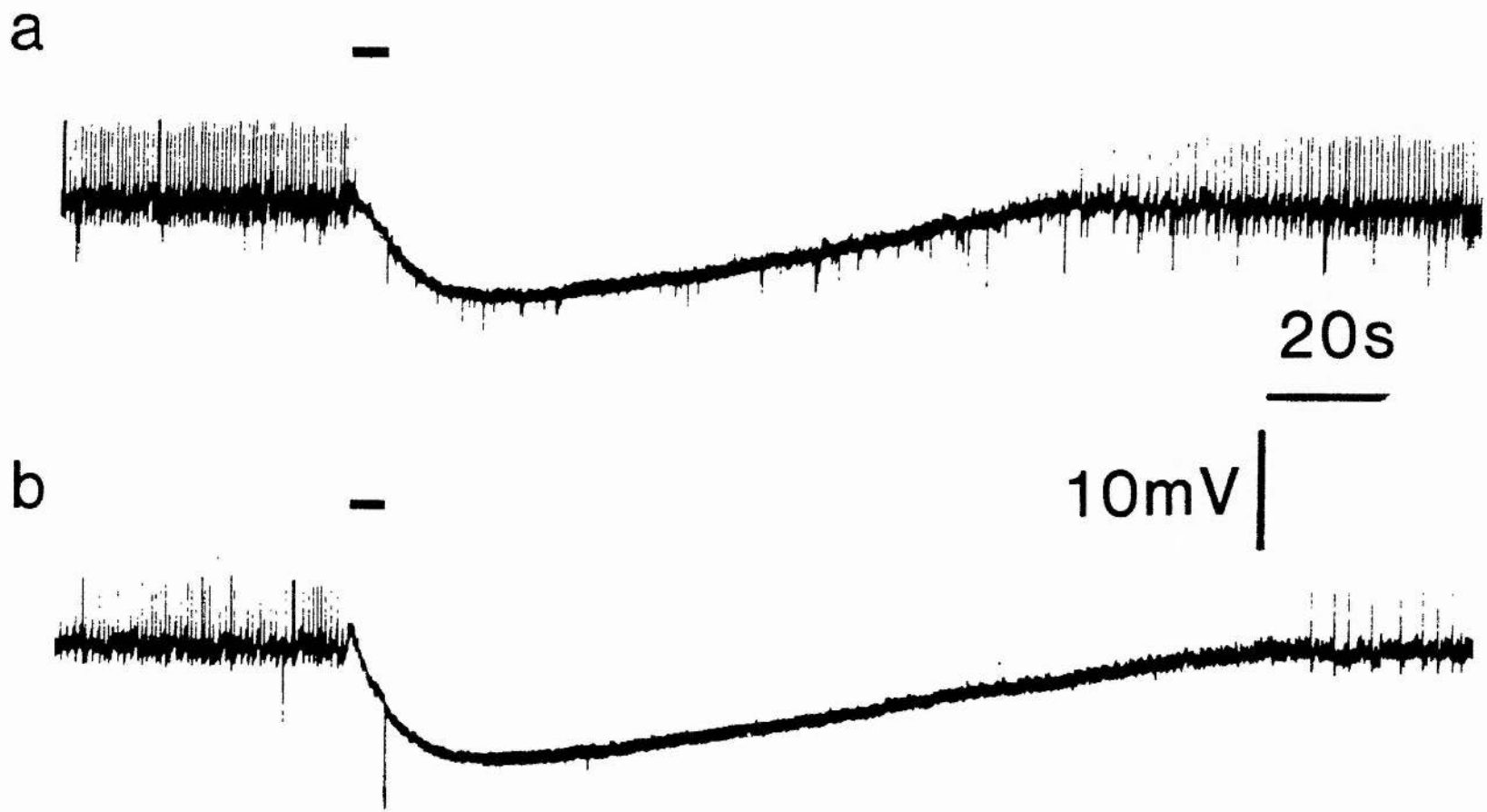

a
20s
b
10mV

Fig. 5.10 Records from an experiment which showed that l-propranolol (100μM) did not affect the response of a CA1 pyramidal neurone to constant doses of serotonin.

a: The response obtained while the slices were bathed in normal ACSF.

b: The response 18min after the introduction of ACSF containing l-propranolol into the recording chamber.

c: The response after 23min exposure of the slices to l-propranolol and an additional 30min "washing" with normal ACSF.

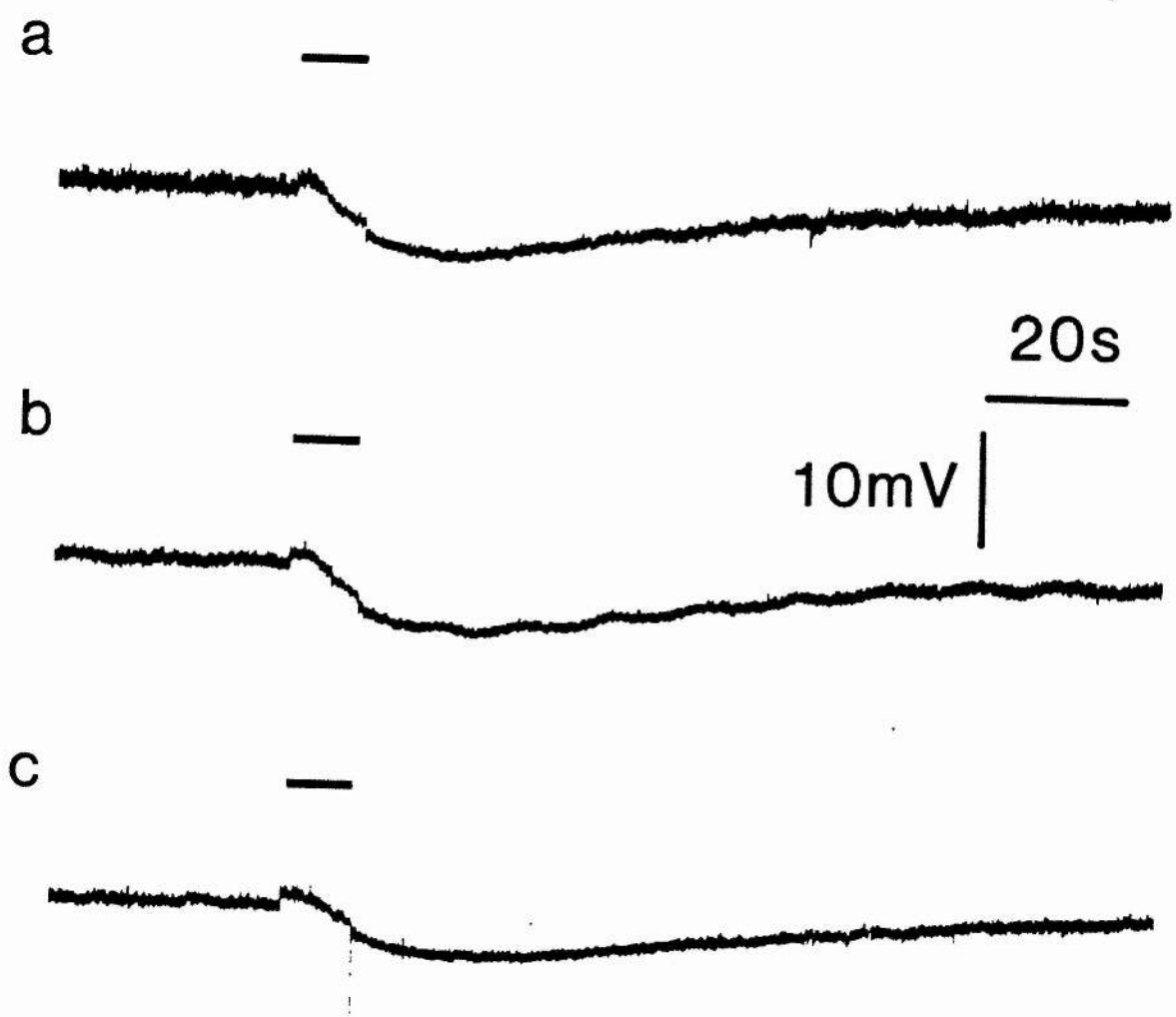

a
20s
b
10mV
c

Fig. 5.11 Records from an experiment which showed that methergoline (100μM) did not affect the response of a CA1 pyramidal neurone to constant doses of serotonin.

a: The response of the neurone while the slice was bathed in normal ACSF.

b: The response of the neurone to the same dose of serotonin 15min after the introduction into the recording chamber of ACSF containing methergoline.

c: The response of the neurone after a further 8min exposure of the slice to ACSF containing methergoline and an additional 10min "washing with normal ACSF.

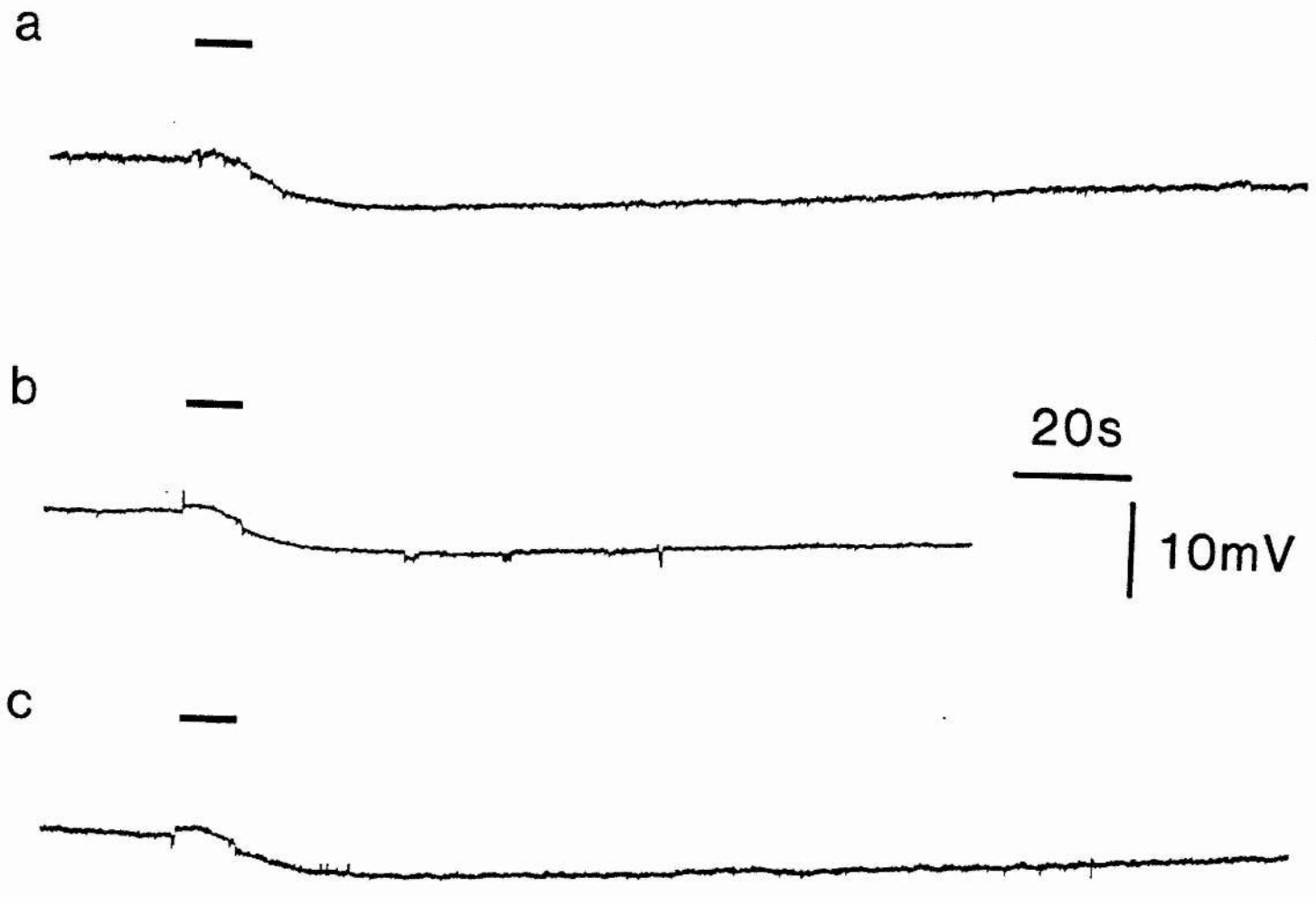
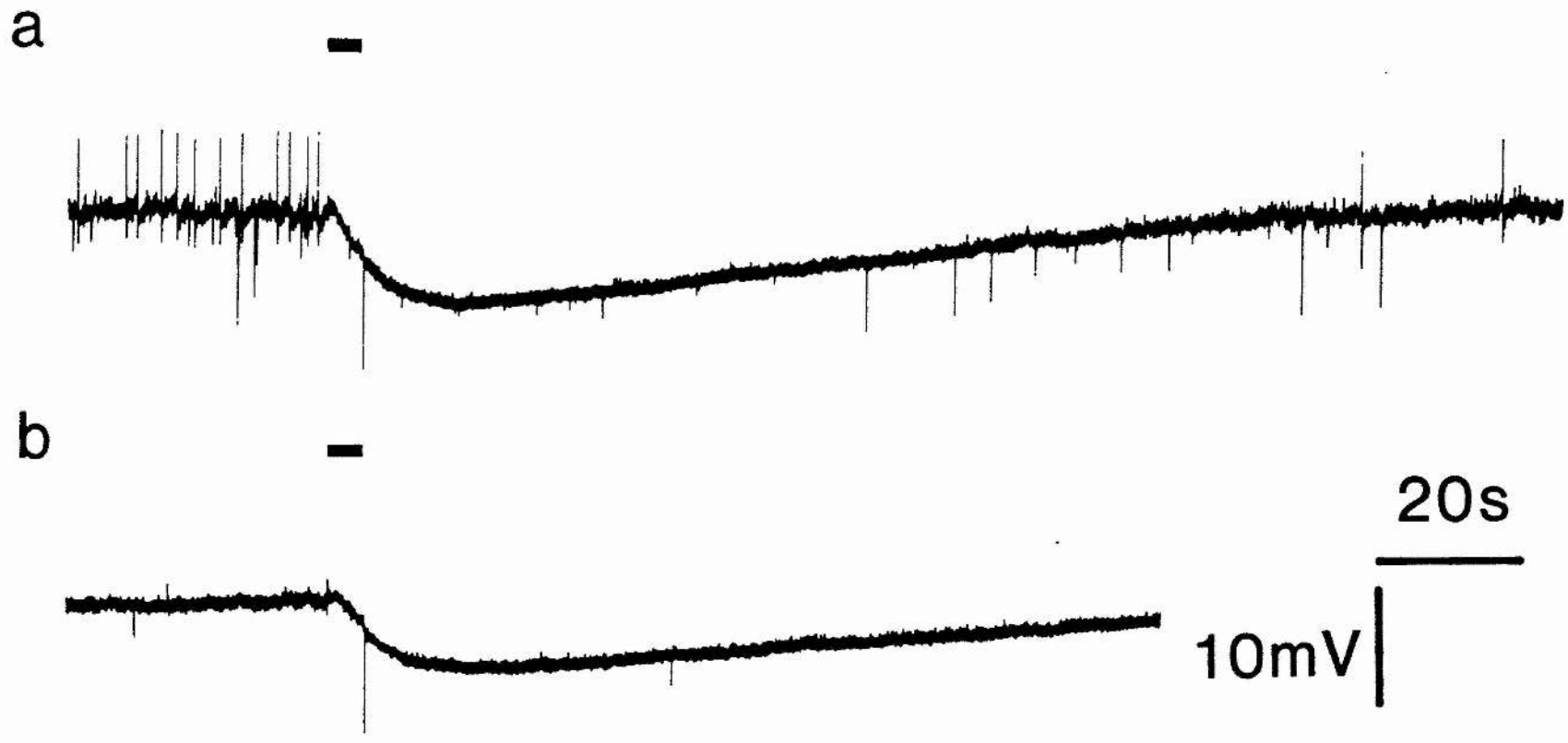

a
b
20s
10mV
c

Fig. 5.12 Records obtained from an experiment which showed that mianserin (100μM) did not affect the response of a CA1 pyramidal neurone to constant doses of serotonin.

a: The response recorded while the slices were bathed in normal ACSF.

b: The response recorded 18min after ACSF containing mianserin was introduced into the recording chamber.


a
b
20s
10mV

(100uM) produced a reduction of the spontaneous spike frequency in all experiments.

l-Propranolol (50μM and 100μM), mianserin (100μM), methergoline (100μM), and cyproheptadine (100μM) reduced the spontaneous firing rate of some neurones. However these putative central antagonists did not produce a substantial blockade of the serotonin response (Figs. 5.9, 5.10, 5.11 & 5.12).

5.5 THE EFFECTS OF OTHER AGONISTS

5.5A Glutamic Acid.

A higher proportion of neurones tested responded to glutamic acid than to serotonin. This difference was probably due to the better current passing characteristics of pipettes filled with glutamic acid solutions.

When applied to a neurone at the resting potential, glutamate produced a depolarisation of up to 5mV. The onset of the response was within 2s of the start of the ejection current pulse. After termination of the pulse, the potential returned to the resting level within 5s. The depolarisation was often sufficient to generate spikes. The amplitude of the response was increased by hyperpolarisation of the neurone.

Fig. 5.13 Records showing the direct and indirect effects of iontophoresed acetylcholine (ACh) on a CA1 pyramidal neurone. The same dose of ACh was given in each case as shown by the bar above the potential trace.

a: The effect of ACh on the neurone in while the slice was bathed in normal ACSF. The burst of i.p.s.p.'s probably resulted from the excitation by acetylcholine of a basket cell which made inhibitory contacts with the impaled neurone. The subsequent depolarisation and increased firing rate were the direct effects on the impaled neurone.

b: The effect of ACh on the neurone 12min after ACSF containing 10mM Mg^{++} was introduced into the recording chamber.

c: The effect of ACh after "washing" the slices with normal ACSF.

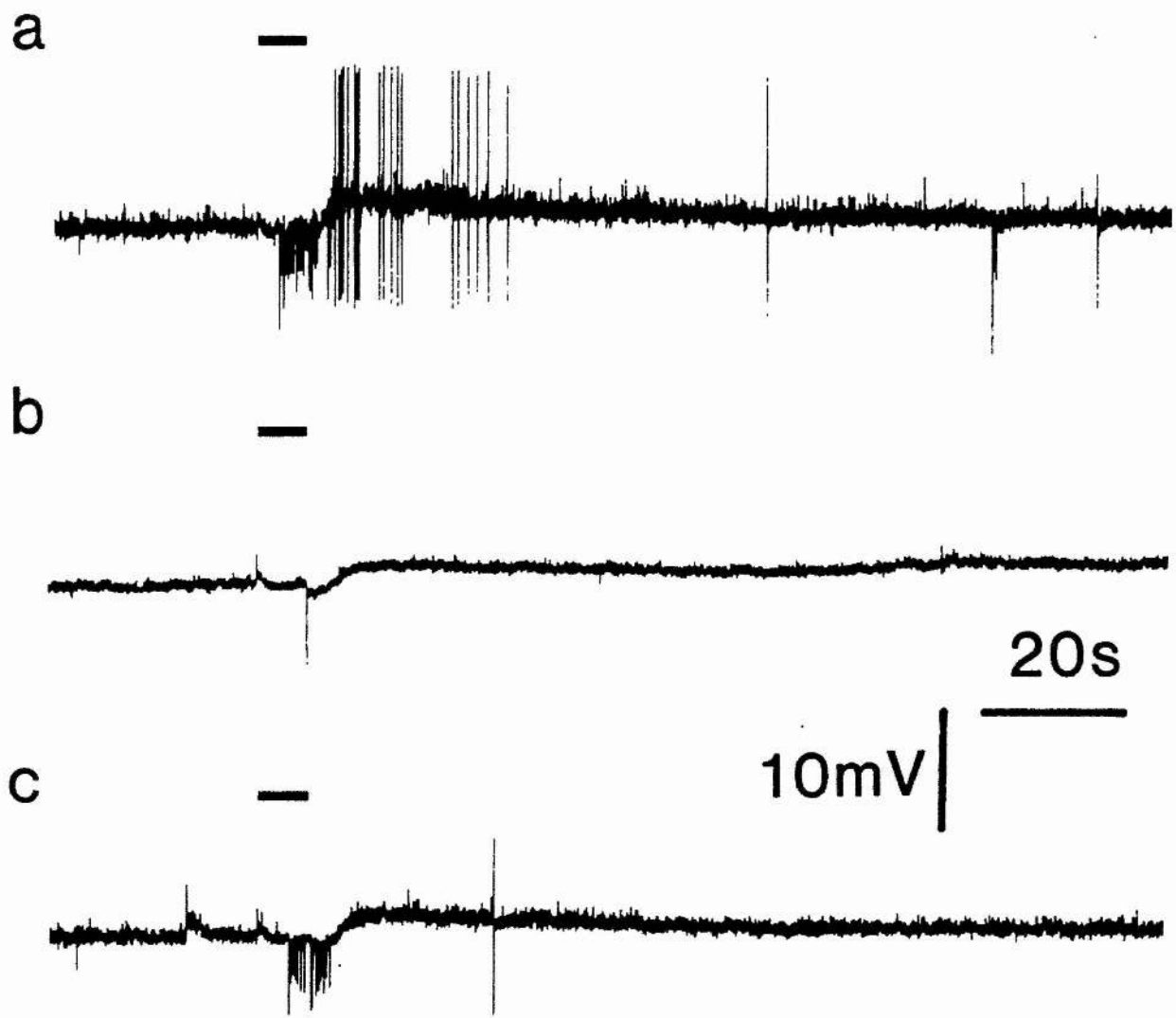
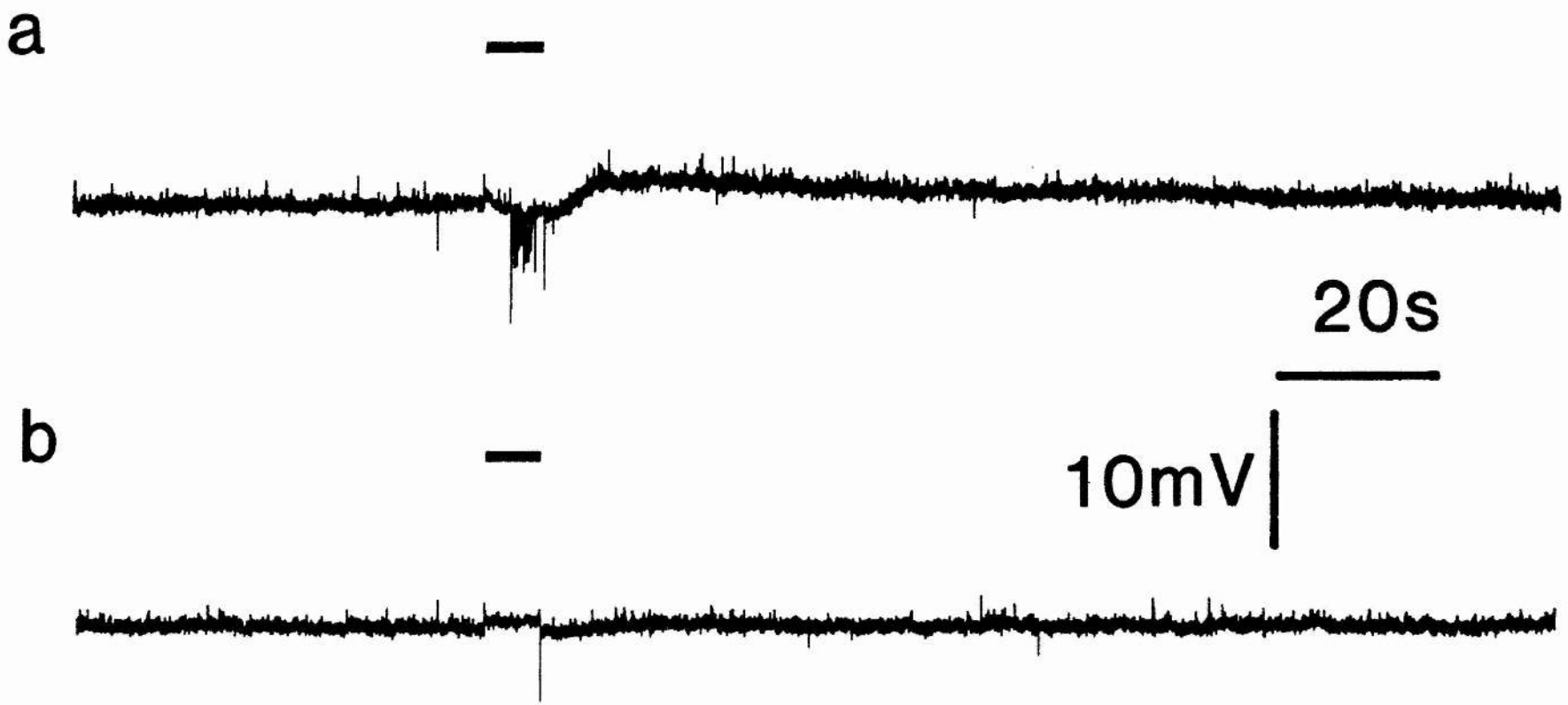

a
b
c
20s
10mV

Fig. 5.14 Records to show that atropine (50μM) blocks the indirect and direct effects of acetylcholine on the same neurone from which the recordings for Fig. 5.13 were obtained.

a: The ACh response recorded while the slice was bathed in normal ACSF.

b: The ACh response 8min after the introduction of ACSF containing atropine into the recording chamber.


a
20s
b
10mV

5.5B Acetylcholine.

At the resting potential, acetylcholine produced a small depolarisation and an increased spike frequency (Fig. 5.13). The onset of the response was within 5s of the start of the current ejection pulse. After the pulse was terminated, about 30s was required for the potential to return to the resting level. The effect on the spike frequency was increased if the neurone was depolarised by about 10mV. Acetylcholine produced no significant effect on the input resistance.

In one experiment, the depolarisation induced by acetylcholine was preceded by a burst of i.p.s.p.'s (Fig. 5.13). These synaptic potentials were abolished when the magnesium concentration of the ACSF was raised to 10mM but the depolarisation was unaffected. In contrast the i.p.s.p.'s and the depolarisation were irreversibly blocked by atropine (50μM) (Fig. 5.14).

CHAPTER SIX

DISCUSSION

6.1 CONDITION OF SLICES AND PROPERTIES OF IMPALED NEURONES

It was important that the slices were in good condition and that the properties of impaled neurones were similar to those previously recorded *in vivo*, and *in vitro* by other workers. In the first experiments, good recordings were not made as: 1) slices tended to dry out and to move in the recording chamber, and 2) slices were prepared at a temperature that was too high (37°C). Better, satisfactory recordings were made routinely after these problems were overcome and after the K^+ concentration in ACSF was decreased and the Ca^{++} concentration was increased. The properties of the CA1 neurones in this study were similar to those found previously *in vivo* (Kandel, Spencer & Brinley, 1961; Kandel & Spencer, 1961; Spencer & Kandel, 1961, a; Spencer & Kandel, 1961, b) and *in vitro* (Schwartzkroin, 1975; 1977), and are compared in Table 6.1.

Neurones could be impaled for up to 9.5hrs without deterioration of the resting potentials, and satisfactory penetrations of neurones could be made up to 14hrs after the animals were sacrificed. In earlier studies by other workers, the recorded resting potentials decreased within a few minutes after penetration, and good impalements could not be made within a few hours of preparing the brain slices (Chuh Loh & McIlwain, 1957; Hillman & McIlwain, 1961; Gibson & McIlwain, 1965). In one of these studies, it was shown that the intracellular K^+ concentration in these slices was greatly reduced during incubation (Gibson & McIlwain, 1965). The low recorded resting potentials and

	Resting Potential		Action Potential		Input Resistance
	min	mean	min	mean	mean
A	-36	-62 ± 2.1	45	70 ± 4.9	13
B	-40	-54.3 ±11.7	45	60 ± 11	16.3
C	-45	-63.4 ± 8.7	45	72.5 ± 8	16.5

Table 6.1 Comparison of the minimum (min) and mean values of the amplitudes of the resting potentials, action potentials, and input resistances of hippocampal neurones in three separate studies. The amplitudes of the potentials are given in mV, and of the input resistance in MΩ. The standard deviation from the mean for the resting and action potentials are also given.

A: Studies on CA3 & CA4 pyramidal neurones in cats in vivo, (Kandel, Spencer & Brinley, 1961; Spencer & Kandel, 1961,a).

B: Studies on CA1 pyramidal neurones in guinea pig hippocampal slices (Schwartzkroin, 1975; 1977).

C: From the present study on CA1 pyramidal neurones in rat hippocampal slices.

decreased intracellular K^+ levels indicated that the slices deteriorated rapidly after preparation. In the present study, measurements of the K^+ concentration in the slices were not made, but the satisfactory, long lasting impalements showed that the slices were maintained in good condition in the recording chamber.

The satisfactory resting potentials, and the potential increase following penetration, indicated that the membrane of impaled neurones sealed around the electrode tip after penetration. The increased stability of the resting potentials, and the larger number of neurones that could be satisfactorily penetrated when the Ca^{++} in ACSF was 4mM, indicated the importance of Ca^{++} for membrane sealing.

Since synaptic potentials were recorded from many neurones, the synaptic mechanisms in the slices would appear to be normal. The frequently observed rapid i.p.s.p.'s were probably the input from the basket cells and these have been shown to be mediated by chloride ions in vivo (Eccles, Nicoll, Oshima & Rubia, 1977).

When the K^+ concentration in ACSF was raised, the resting potentials of impaled neurones were decreased, in a reversible manner, over a period of about 10min. However, the potential changes produced by alterations of the K^+ concentration were not as great as expected. In addition, increasing the K^+ concentration also produced an increased firing rate of the impaled neurones. The frequency of synaptic potentials, particularly of i.p.s.p.'s, was increased by raising the K^+ concentration in the

ACSF. This change might have been due to an increased firing rate of other neurones in the slice, or the revelation of these potentials by the depolarisation produced by increasing the K^+ concentration.

TEA increased the duration of single action potentials, and successive action potentials in a train of impulses were increasingly affected. In addition, the resting potential of impaled neurones was decreased by TEA but the frequency of synaptic potentials was increased. It therefore appeared that TEA might be acting on two types of K^+ channel. First, TEA sensitive K^+ channels that were not voltage dependent: blockade of these channels decreased the membrane potential of the impaled neurone, and of other neurones in the slice so that their impulse frequency increased and the synaptic potential frequency was increased. Blockade of these channels also produced some prolongation of the action potential. Second, TEA sensitive voltage dependent K^+ channels: blockade of these channels only occurred after channel activation during impulse activity so that successive spikes in a train became longer as this blockade was effected. A similar fall of the resting potential and prolongation of the action potential was observed by Schwartzkroin & Prince (1980) after intracellular injection of TEA. The effects of TEA applied extracellularly and intracellularly were the same except that: intracellular TEA had no effect on the synaptic potentials whereas synaptic potentials were more frequent when TEA was applied extracellularly.

The effects of increasing the K^+ concentration, and of

adding TEA to the ACSF, showed that the ACSF in the recording chamber was in equilibrium with the extracellular fluid of the slices. These experiments also showed that about 10min was required for the extracellular fluid in the slices to equilibrate with the ACSF in the recording chamber. A similar time course for equilibrium to be reached has been found in studies on cortical slices for K^+ (Gibson & McIlwain, 1965), and for Mg^{++} and Ca^{++} (Richards, 1971)and in hippocampal slices for K$D+$U (Benninger, Kadis & Prince, 1980). Since the time course of equilibration was known, it was expected that other, larger ions and molecules would penetrate slices in approximately the same period of time.

6.2 IDENTITY OF IMPALED NEURONES

Satisfactorily impaled neurones were not identified by antidromic or orthodromic stimulation as previously described in vivo and in vitro (Kandel, Spencer & Brinley, 1961; Schwartzkroin, 1975). Instead some neurones were impaled and injected with the fluorescent dye Lucifer Yellow (Stewart, 1978; 1981). The major advantages of this particular dye were: 1) its high fluorescence intensity, 2) its rapid spread through neuronal processes, 3) the short injection period and low injection current required to fill neurones with dye, and 4) injected neurones could be examined in intact slices. Dye injections allowed positive identification of the impaled neurones, and, in addition, the integrity of the neurones could be checked. Only slices transverse to the hippocampal axis would be expected to contain intact pyramidal

neurones and granule cells.

All neurones had low resting potentials when impaled on electrodes containing Lucifer Yellow. These low potentials were probably not due to the dye itself: normal resting potentials were recorded during dye injection into molluscan central neurones and neurones of the turtle retina (Stewart, 1978), and into crayfish neurones (Miller & Selverston, 1979), normal resting and action potentials were recorded during dye injection for long periods of time. Since the laboratory lighting and the direct illumination of the slices was reduced during injections, the low potentials were not produced by the toxic effects of the dye in injected neurones exposed to light (Miller & Selverston, 1979). It is more probable that the LiCl solution, in which the dye was dissolved, produced these low potentials. Reduced resting and action potentials were also recorded from neurones of the cerebral cortex in cats in vivo (Takato & Goldring, 1979), and from guinea pig CA3 hippocampal pyramidal neurones in vitro (MacVicar & Dudek, 1980, b) when these neurones were impaled on electrodes containing LiCl/Lucifer Yellow solution.

Injected neurones in the CA1 and CA3 regions were all identified as pyramidal neurones. In the area dentata, injected neurones were identified as granule cells. The basket cells, the type of interneurone seen by Schwartzkroin & Mathers (1978), and other neurones were not seen.

Pyramidal neurones were identified from the position of their

perikarya, and the projections of their apical and basal dendrites. The structure of the injected pyramidal neurones was similar to that shown by silver impregnation techniques (Cajal, 1911; Lorente de No, 1934), but there was one major difference: dendritic spines were not observed. These spines appear to be a normal feature of pyramidal neurones and have been shown to be present after incubation of slices (Harris, Cruce, Greenough & Teyler, 1980). Failure to observe spines on dye injected neurones might have been due to the "halo" effect produced by Lucifer Yellow: the high fluorescence intensity of the dye produced a "halo" around the edge of dye filled structures. However dendritic spines were observed despite a "halo" effect on CA3 pyramidal neurones after injection of Lucifer Yellow in vitro (MacVicar & Dudek, 1980, b). It is more probable that for some unknown reason, dendritic spines on the neurones were not filled with dye in the present study.

Two types of CA1 pyramidal neurone were distinguished on the branching of the apical dendrite. Whether such a division is real, or the result of Lucifer Yellow not entering all neuronal processes, or an artefact produced by the post-injection processing was not clear. Perhaps the injected neurones represent a range of neurones in which there are between one and several major apical dendrites. Although drawings by Cajal (1911) and by Lorente de No (1934) show some pyramidal neurones with more than one major apical dendrites and some with only a single major dendrite, no division of pyramidal neurones into different populations was made by these workers on this basis.

The axons of CA1 neurones were traced caudally in the slices: this projection is the same as that shown by antidromic stimulation in vivo and in vitro (Andersen, Bland & Dudar, 1973; Schwartzkroin, 1975). However, Cajal (1911) and Lorente de No (1934) described axons from these neurones projecting towards the fimbria: this projection was only seen in one neurone after dye injection, and has not been shown physiologically (Andersen, Bland & Dudar, 1973). In contrast, all identified axons of CA3 pyramidal neurones were traced towards the fimbria in the same way previously described in anatomical and physiological studies (Cajal,1911; Lorente de No, 1934; Andersen, Bland & Dudar, 1973). The role of the short branches of the axons of CA1 and CA3 pyramidal neurones is not known. In both regions, some of these collaterals probably make synaptic connections locally with basket cells. In the CA3 region, some of the collaterals probably form the longitudinal association pathway (Lorente de No, 1934; Hjorth-Simonsen, 1974), and others may make connections locally with other pyramidal neurones within the lamellar of the slice (MacVicar & Dudek, 1980a). In the CA1 region, a longitudinal pathway between lamellae and local connections between pyramidal neurones in the same lamellar have not been described.

Movement of Lucifer Yellow between neurones of the turtle retina, some of which have been shown to be electrically coupled, has been shown by Stewart (1978). However the spread of dye between hippocampal neurones was not expected. There are three possible explanations for two neurones being filled with dye after an injection. First, leakage of dye from one neurone into the

extracellular space and its subsequent uptake into the second neurone. This was not likely since dye was never observed in the extracellular space, and Lucifer Yellow has been shown not to be taken up by neurones (Stewart, 1978). Second, movement of the electrode from one neurone to the other during injection. This explanation was also unlikely since on every occasion that impalement of a neurone was disrupted, a second neurone was never penetrated. Third, and the most probable explanation, the direct movement of the dye from one neurone to the next. The route for spread of dye between neurones is not clear. Lucifer Yellow has been shown to move across gap junctions in embryonic preparations (Bennett, Spira & Spray, 1978). The evidence that all neurones filled with dye after a single injection were equally fluorescent supported the possibility that Lucifer Yellow moved through such junctions in this study. However, gap junctions have not been described in the hippocampus. It is also possible that the dye moved across synaptic gaps in a retrograde manner as reported for movement of Procion Yellow between neurones (Kuhnt, Kelly & Schaunberg, 1979). However, the retrograde movement of Procion Yellow required repeated synaptic stimulation: in this study, neurones were not stimulated synaptically at all. Whether dye coupling between CA1 pyramidal neurones was indicative that electrical coupling also occurs between these neurones is not known. However dye coupling with Lucifer Yellow between CA3 pyramidal neurones has also been observed by MacVicar & Dudek (1980, b), and these workers have also found that some CA3 pyramidal neurones are electrically coupled (MacVicar & Dudek, 1980, a).

6.3 THE SEROTONIN RESPONSE OF CA1 PYRAMIDAL NEURONE

CA1 pyramidal neurones were hyperpolarised, and their firing rates were reduced by iontophoresed serotonin. This response is consistent with reduced firing rates produced by serotonin *in vivo*, and recorded extracellularly, in the hippocampus of rats (Segal, 1975) and in the other brain nuclei of rats and in the brain nuclei of other mammals (Bloom, Hoffer, Siggins, Barker & Nicoll, 1972). The hyperpolarisation appeared to be a direct effect of serotonin, and was not mediated by excitation of inhibitory baskets cells in the slices by serotonin. The evidence for the effect of serotonin being direct was: 1) a burst of i.p.s.p.'s were never observed after application of serotonin, and 2) the response was independent of the Cl^- concentration of ACSF. Activation of the basket cells by serotonin would have produced a burst of i.p.s.p.'s similar to that recorded from one neurone after application of acetylcholine. The hyperpolarisation was different to that produced by -aminobutyric acid (GABA) when applied to the perikarya of pyramidal neurones *in vitro* (Andersen, Dingledine, Gjerstaad, Langmoen & Laursen, 1980). The serotonin response was slow on onset and on recovery, but the effect and recovery from the effect of GABA were rapid.

The hyperpolarisation produced by serotonin was in association with a reduction of the input membrane resistance. This change did not result from an increased membrane permeabilty to Cl^-: when the Cl^- concentration in ACSF was changed, the response was not altered. However the amplitude of the response was dependent on

the extracellular K^+ concentration. The experiments to determine the reversal potential also indicated that K^+ efflux, rather than Cl^- influx, produced the hyperpolarisation brought about by serotonin. The increased membrane conductance, and the K^+ dependency of the slow hyperpolarisation produced by serotonin was similar to the serotonin induced type "B" response recorded from some molluscan central neurones (Gerschenfeld, 1971; Gerschenfeld & Paupardin-Tritsch, 1974). In addition, since the response was reversible by membrane hyperpolarisation, it was evident that the serotonin induced hyperpolarisation was not the result of the activation of an ionic pump by serotonin. Such electrogenic ion pumps have been shown to be activated by internal or external ionic changes, but their role in generating synaptic potentials has not been demonstrated (Kehoe & Ascher, 1970).

A similar direct hyperpolarisation of CA1 pyramidal neurones has been described by Jahnsen (1980) on slices prepared from guinea pigs, and by Segal (1980) on slices prepared from rats. Serotonin was shown to depolarise some guinea pig pyramidal neurones (Jahnsen, 1980), but this type of response was not described by Segal (1980) and was not seen in the present study.

Rapid potential changes associated with the interactions of a transmitter and postsynaptic membrane receptors are probably due to conformational changes of specific ion channels produced by these interactions. A change of channel conformation produces a change in the membrane permeability. Such membrane changes may be associated with the rapid, Na^+ dependent depolarisation of muscle

end-plates by acetylcholine, and the rapid, Cl^- dependent hyperpolarisation produced by acetylcholine in molluscan neurones and by GABA in the mammalian CNS. Slower potential changes such as those seen in the present study, have been postulated to be the result of membrane permeability changes brought about indirectly by the interaction of a transmitter with its receptors. A role for the cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as "second messengers" between the interaction of a transmitter and its receptors, and the change of membrane permeability has been suggested.

The best evidence that cAMP mediates serotonin induced changes of membrane permeability comes from studies on molluscan neurones. At depolarised levels only, serotonin produced a slow, inward Ca^{++} current in some Aplysia neurones (Pellmar & Wilson, 1977; Pellmar & Carpenter, 1979) or a slow, inward K^+ current in some neurones of Helix (Deterre, Paupardin Tritsch, Bockaert & Gerschenfeld, 1981). Intracellular injection of cAMP also produced these responses, and the cAMP induced currents were voltage dependent in the same way as the serotonin responses (Pellmar, 1981; Deterre, Paupardin Tritsch, Bockaert & Gerschenfeld, 1981). An inward current has also been demonstrated in the A neurone of the buccal ganglia following stimulation of the giant serotonin neurones of the cerebral ganglia in Helix: the synaptic response is also only observed at depolarised levels (Cottrell, 1981).

The voltage dependent serotonergic effects on the Ca^{++} and K^+ conductances are particularly interesting and may provide

explanations for the phenomenon of presynaptic facilitation. Transmitter release is triggered by a Ca^{++} influx which occurs during invasion of action potentials into neuronal terminals. Serotonin may act to increase the voltage dependent increase of Ca^{++} conductance, increase the amount of transmitter released from the terminals, and therefore facilitate the activity of the presynaptic neurones. Such an increase of voltage dependent Ca^{++} conductance may be mediated by cAMP as suggested by Klein & Kandel (1978) in the case of serotonergic facilitation of the gill withdrawal reflex in Aplysia. A similar increase of the amount of transmitter released would occur where serotonin reduced the voltage dependent K^{+} conductance at neuronal terminals. Increasing the duration of action potentials would increase the Ca^{++} influx which triggers transmitter release.

Whether cyclic nucleotides mediate transmitter effects in the mammalian CNS is not clear. There is evidence that cAMP may mediate some serotonin and noradrenaline effects (see Bloom, 1978). Cyclic GMP has also been suggested to be a "second messenger" in the long lasting depolarisation associated with a decreased membrane conductance produced by acetylcholine in cortical neurones. Since both cAMP and cGMP were possible "second messengers", it was suggested that membrane permeability may be controlled by both cAMP and cGMP concentrations rather than the concentration of either nucleotide in a neurone: the so called Yin-Yang hypothesis (Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl & Estensen, 1975; Stone, Taylor & Bloom, 1975).

If such a hypothesis is correct, then membrane hyperpolarisation associated with an increased K^+ conductance may be produced by a change of the cAMP/cGMP ratio. The opposite change in the cAMP/cGMP ratio might produce a membrane depolarisation associated with a decreased K^+ conductance. Therefore two transmitters could have opposing effects on a membrane but these effects could be mediated by a common pathway within neurones. In the hippocampus, for example, acetylcholine produces a depolarisation and decreased membrane conductance associated with an increased exciteability in vitro (Dodd, Dingledine & Kelly, 1980). However, serotonin produces an hyperpolarisation and an increased conductance associated with decreased exciteability. In addition, a transmitter may have opposing effect in two different brain nuclei. In the hippocampus, serotonin may act as a neuromodulator to decrease the exciteability of pyramidal neurones: an exact opposite effect to that shown recently by VanderMaelen & Aghajanian (1980) in the facial motor nucleus where serotonin facilitates excitation of spinal motorneurones.

In the experiments to determine the reversal potential, two unexpected observations were made. First, the response amplitude relationship of the membrane potential and the response amplitude was not linear. Second, it was noticed that the maximum potential change produced by serotonin at high potentials occurred later than the change at lower potentials. The non-linearity of the response at potentials more negative than the reversal potential might be explained by an insufficient amount of extracellular K^+ to carry

the inward potassium current. However when the second observation is also considered, there are two other explanations. First, that the interaction between serotonin and its receptors on the membrane surface is reduced at high potentials, so that at high potentials the response was not as great as expected. Second, that the membrane channels that are activated following the interaction of serotonin and its receptor are voltage dependent, so that at higher potentials, fewer channels were opened or channels were opened more slowly. Marked voltage dependency of acetylcholine activated Na^+ channels has been demonstrated in molluscan neurones, and neurones in rat sympathetic ganglia (Ascher, Marty & Neild, 1978; Ascher, Large & Rang, 1979). In these studies, it was shown that the kinetics of the acetylcholine activated Na^+ channels were altered by changes of membrane potential. Whether the characteristics of the serotonin activated K^+ channels in hippocampal neurones were voltage dependent could not be examined in the present study since voltage clamp techniques were not used.

Consistent with the observations made by Segal (1980) and by Jahnsen (1980), the response was best recorded when serotonin was applied close to the s. pyramidale. This is in contrast to the distribution of serotonergic terminals in the hippocampus which is mainly to the s. lacunosum and the s. moleculare (Moore & Hilaris, 1975; Azmitia & Segal, 1978). However it has been shown that the increase of the extracellular K^+ produced by local application of serotonin was greatest when serotonin was applied to the s. moleculare (Segal & Glutnick, 1980). The difference between data obtained by intracellular recording techniques and that from

measurements of K^+ concentration may be explained in two ways. First, the potential changes produced by serotonin on the distal dendrites of a neurone might not be passively conducted as far as the recording site in the perikaryon. Recently it has been shown that, in CA3 pyramidal neurones, attenuation of a potential change between the tip of the dendrites and the perikaryon of a neurone is probably small (Johnston, 1981). If the CA1 pyramidal neurones have similar passive cable properties, this explanation is not acceptable. Second, the extracellular K^+ concentration is dependent on the local extracellular volume which is not known: it is possible that a large concentration increase could be due to a large efflux of K^+ into a large extracellular volume, or a small K^+ efflux into a small volume.

The data obtained in this study on the effects of serotonergic antagonists was clear. Methysergide blocked the response in an irreversible manner. A similar but reversible blockade of the serotonin response of the pyramidal neurones in vitro has been observed by Segal (1980) and has also been previously described in vivo (Segal, 1976). The blockade of the serotonin response by methysergide, and the blockade of the acetylcholine response by atropine, showed that molecules with a molecular weight of up to 300 entered the slices after introduction into the recording chamber in ACSF. The time course of the blockades produced by methysergide and atropine were similar: after introduction into the recording chamber, the response was partially blocked in 7min and completely blocked after 15min.

In contrast, cyproheptadine, methergoline, mianserin, and l-propranolol did not affect the serotonin responses of the pyramidal neurones. These data are similar to those obtained for the potency of cyproheptadine, methergoline and mianserin on the serotonin response of these neurones in vivo (Segal, 1976).

However the data from the behavioural studies (Corne, Pickering & Warner, 1963; Green & Grahame-Smith, 1976; Costain & Green, 1978; Fuxe, Ogren, Agnati & Jonsson, 1978; Weinstock & Weiss, 1980; Matthews & Smith, 1980; Sloviter, Drust, Damiano & Connor, 1980), and from the ligand binding studies (Bennett & Snyder, 1976; Middlemiss, Blakeborough & Leather, 1977; Fuxe, Ogren, Agnati & Jonsson, 1978; Peroutka & Snyder, 1979) indicated that cyproheptadine, methergoline, methysergide, mianserin, and l-propranolol are potent inhibitors of serotonin in the CNS. Recently, evidence for a number of different types of serotonergic receptor has been obtained from ligand binding studies (Bennett & Snyder, 1976; Peroutka & Snyder, 1979). It is therefore possible that in the hippocampus there is a particular population of serotonergic receptors which are found in small numbers elsewhere in the brain or only in some other brain nuclei. It would therefore be interesting to investigate the potency of these antagonists in other brain nuclei which receive a serotonergic input from the raphe nuclei and which are amenable for slice studies. Nuclei such as the lateral geniculate nucleus and the hypothalamus might be considered good candidates for such a study.

It would also be interesting to examine the effects of the

five putative serotonin antagonists used in this study on serotonin responses after chronic administration. There is evidence from behavioral studies that long term administration of cyproheptadine or methysergide induces supersensitivity of serotonergic receptors (Jones, 1980). This supersensitivity was demonstrated by an increased duration of serotonin induced sleep in chicks and was also produced by tricyclic antidepressants. Supersensitivity of serotonin receptors has recently been demonstrated electrophysiologically in vivo following chronic administration of tricyclic antidepressants (DeMontigny & Aghajanian, 1978). The delayed supersensitivity to serotonin may explain the delayed action of these antidepressants in the clinical situation.

This study has shown clearly one central action of serotonin and its mechanism of action. The hippocampal slice preparation may be useful for further study of the mechanisms of serotonin action, of putative serotonergic antagonists and analogues on central neurones.

APPENDIX

PROPRANOLOL AS A SEROTONIN ANTAGONIST IN THE SNAIL

INTRODUCTION

Before the investigation of the actions of serotonin on neurones in rat hippocampal slices was started, a short study was made on the effects of propranolol on some serotonin responses in the snail, Helix. This study was made to determine: 1) whether propranolol blocked serotonin responses in the snail, and 2) whether d-propranolol and l-propranolol were equipotent as antagonists of serotonin. Two preparations were used: first, the isolated ganglia of the central nervous system, and second, the isolated and perfused heart.

The central nervous system of the snail consists of three ganglionic masses: the paired cerebral ganglia, the paired buccal ganglia, and the visceral ganglion. In each cerebral ganglion, there is a giant serotonin neurone (G.S.N.) which makes synaptic contacts with neurones in the buccal ganglia (Cottrell, 1970). In each buccal ganglion, there are two neurones, the M and P cells, that have been shown to be depolarised following stimulation of a G.S.N. or local application of serotonin (Cottrell, 1970; Cottrell & Macon, 1974). Other neurones, in the visceral ganglion, have also been shown to be depolarised by serotonin, but serotonin has been shown to hyperpolarise some neurones (Gerschenfeld & Paupardin Tritsch, 1974).

Serotonin has been shown to be excitatory on the heart of many molluscs (Welsh, 1957; Kerkut & Cottrell, 1963). On the perfused snail heart preparation, serotonin produces positive inotropic and

chronotropic effects. That is the amount of contraction of the heart and the frequency of these contractions is increased by serotonin.

METHODS

1) Experiments on Isolated Ganglia.

Experiments were all performed on specimens of Helix pomatia, which were obtained from Haig & Gerrard Ltd., East Preston, Sussex. Snails were kept at 8°C until 24hrs before use when they were transferred to room temperature.

After removing the shell from an animal, the circumoesophageal ring or ganglia were exposed by cutting away the muscle tissue of the body wall. The cerebral, buccal and visceral ganglia were removed with their connectives and were pinned onto soft plastic on the bottom of a small Perspex chamber. The ganglia were superfused with a physiological solution containing: NaCl 80mM, KCl 4mM, $CaCl_2$ 7mM, $MgCl_2$ 5mM, and Tris hydroxymethylamine 5mM (pH 7.8). The perikarya of neurones in the cerebral, buccal or visceral ganglia were exposed by removal of the overlying connective tissue.

Neurones were impaled on double barrelled micropipette electrodes. One barrel was used to record potential: the electrode signal was amplified and displayed on an oscilloscope (Tektronix 502A). Permanent records were made using a pen recorder (Gould, Brush 220). The second barrel was used for intracellular injection of current: steady and pulsed currents could be injected separately or simultaneously.

Serotonin, or in some experiments acetylcholine, was

iontophoresed from a single barrelled micropipette positioned independently of the recording electrode. Serotonin was applied as a solution of serotonin creatinine sulphate (Sigma) in distilled water, and acetylcholine was applied as a solution of acetylcholine chloride (BDH) in distilled water. Serotonin or acetylcholine were applied in constant, submaximal doses, and these doses were applied repeatedly at constant intervals in each experiment.

l-Propranolol and d-propranolol were dissolved in physiological solution and were applied through the superfusion system. In each experiment, both isomers were applied at the same concentration and for the same period of time. An interval of at least 20min separated the application of the two isomers to a single preparation. In some experiments, d-propranolol was applied before l-propranolol, but in other experiments, l-propranolol was applied first.

2) Perfused Heart Experiments.

All experiments were performed on specimens of Helix aspersa which were obtained locally. Snails were kept at 8°C until 48hrs before use when they were transferred to room temperature.

After removing the shell, the mantle and the pericardium were cut away to expose the heart. The atrium was cannulated, and the heart was cut free. The cannula was clamped vertically to a stand and attached to one arm of a Y shaped piece of glass tube. Through the second arm of this tube, physiological solution was supplied at

a constant rate. The physiological solution contained (Meng, 1960): NaCl 59mM, KCl 5.8mM, $CaCl_2$ 10mM, $NaHCO_3$ 13.1mM, and $MgCl_2$ 16mM.

Serotonin was applied as a solution of serotonin creatinine sulphate (Sigma) dissolved in physiological solution. Doses of serotonin were applied via the third arm of the Y shaped tube attached to the cannula. Doses were applied in a volume of 50ul of solution, and were repeated every three minutes. The heart was attached by the base of the ventricle to a light lever so that contractions of the heart could be recorded on a kymograph.

In these experiments, the responses of the heart to a range of doses of serotonin were first recorded while the heart was perfused with normal solution. Then the heart was perfused for 10min with solution containing one propranolol isomer and the doses of serotonin were repeated, and if necessary increased. After "washing" the heart for 10min with normal solution, the heart was then perfused with solution containing the second propranolol isomer for 10min and the doses of serotonin repeated once again. The propranolol isomers were applied in the same concentration in each experiment: in some, d-propranolol was applied first, and in others l-propranolol was applied first.

RESULTS

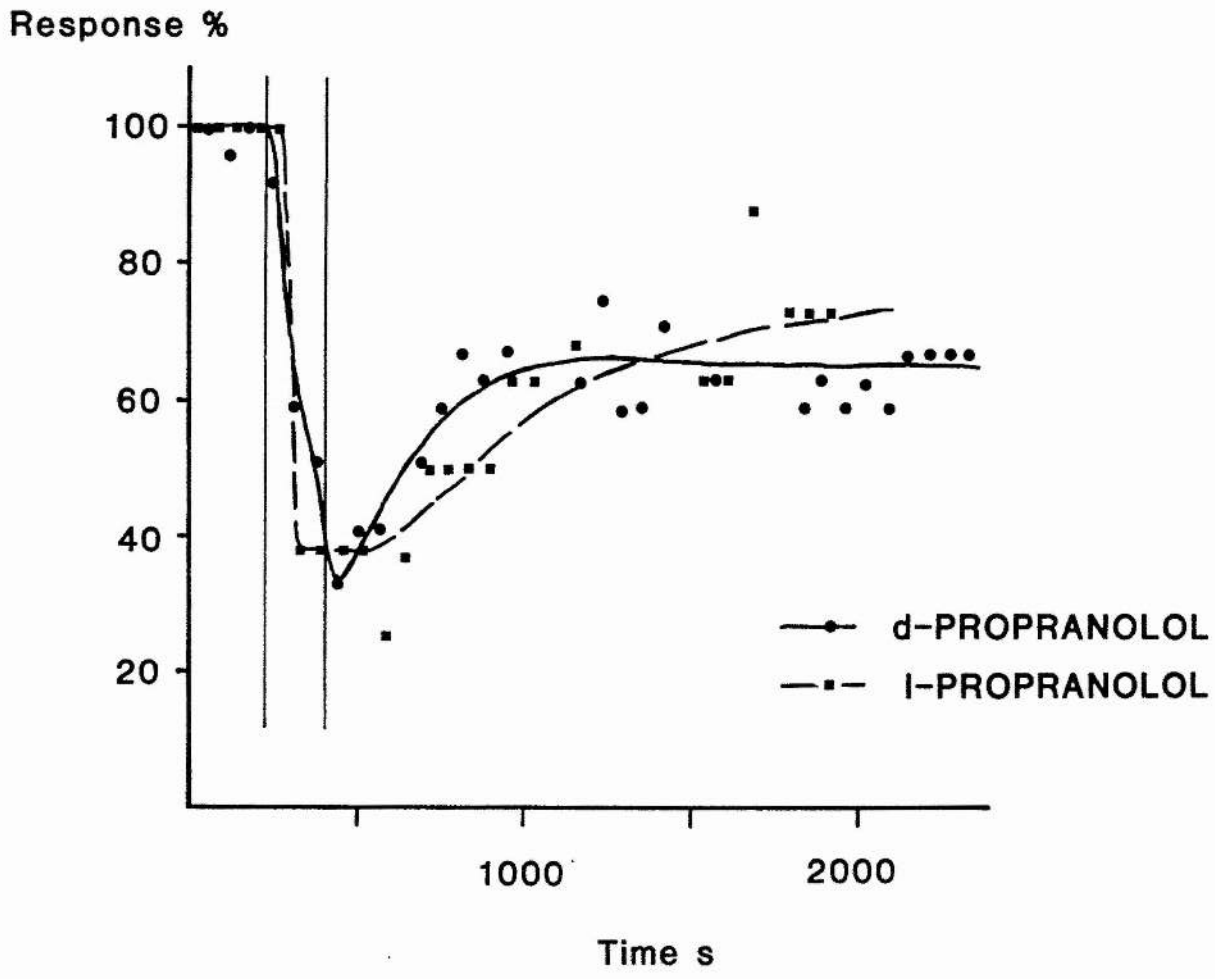
1) Experiments on Central Neurones.

Iontophoresis of serotonin onto the P and M cells of the buccal ganglia, and onto some neurones in the visceral ganglion produced a depolarisation of up to 20mV. The depolarisations were accompanied by a decrease of the input membrane resistance. When the membrane was at or close to the resting potential, serotonin also increased the firing rate of the neurones. When the membrane was hyperpolarised by more than about 7mV, serotonin produced a subthreshold depolarisation.

These depolarising responses were blocked by d-propranolol and by l-propranolol at concentrations between 50μM and 200μM. The responses were reduced by between 10% and 80% by propranolol and the isomers were equipotent (Fig. A.1). The blockade produced by propranolol was not completely reversed by washing the preparation with normal physiological solution, but the response returned to a constant amplitude. However, propranolol was not effective as an antagonist when serotonin produced a hyperpolarisation in some experiments on neurones of the visceral ganglion.

Acetylcholine depolarised and increased the firing rate of some neurones including the P and M neurones of the buccal ganglia. These depolarising responses were also reduced by propranolol in concentrations between 50μM and 200μM: d-propranolol and l-propranolol were equipotent as acetylcholine antagonists.

Fig. A.1 The relationship between response amplitude and time plotted from data obtained during an experiment made to determine the effect of propranolol on the serotonin response of an M neurone. The depolarisation produced by serotonin was reduced by the same extent by d- and l- propranolol (200μM). d- and l- propranolol were applied for the same period of time (3min), as indicated by the vertical bars on the graph; in this experiment, d-propranolol was applied before l-propranolol.


Response %
100
80
60
40
20
1000
2000
Time s
d-PROPRANOLOL
l-PROPRANOLOL

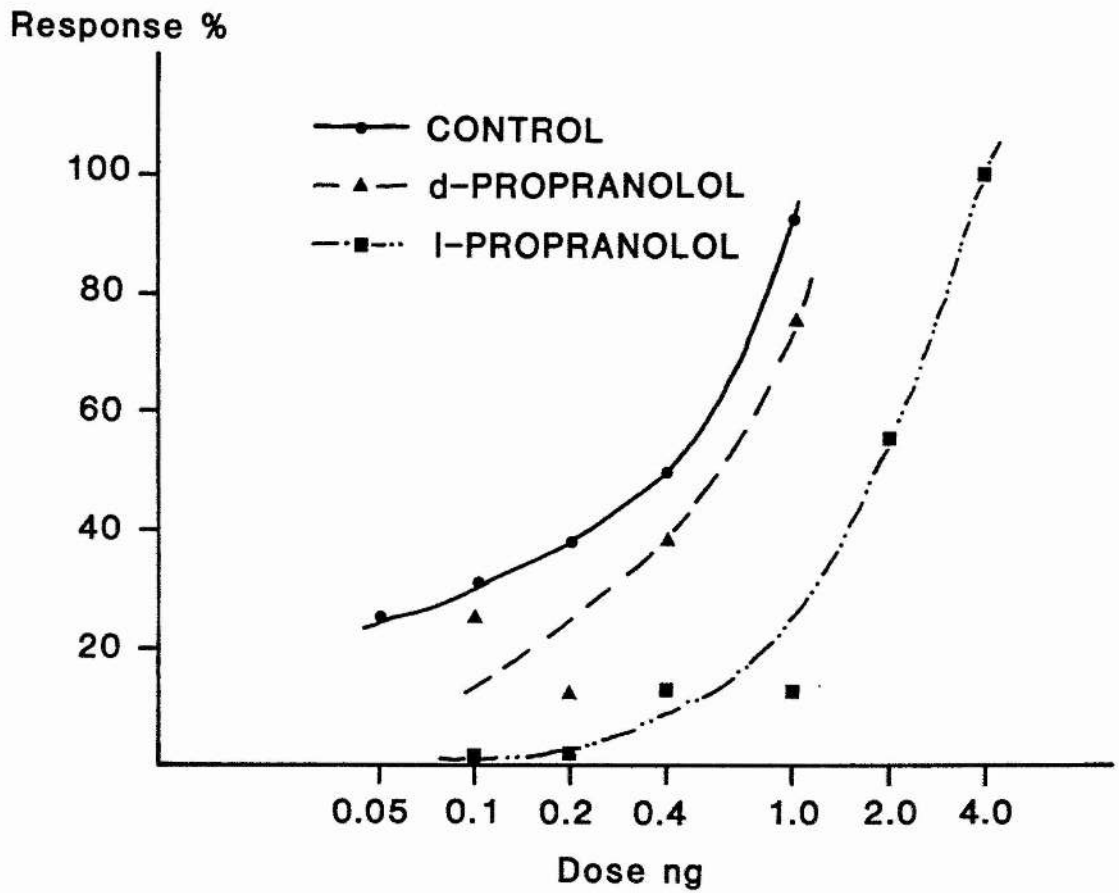
However propranolol was not effective as an antagonist of acetylcholine in those experiments where acetylcholine hyperpolarised the neurones.

2) Experiments on the Perfused Heart.

Serotonin in doses of between 50pg and 4ng increased the amount of contraction and the frequency of contractions of the heart in a dose dependent manner. However the inotropic effect was generally larger and more easily measured than the chronotropic effect. Responses were measured as the difference between the amount of contraction just before a dose of serotonin and at the peak of the serotonin effect. The responses during an experiment were expressed as a percentage of the maximum recorded response in the experiment.

For each experiment, the relationship between the dose of serotonin and the amplitude of the response of the heart was plotted (Fig. A.2). From each graph the dose of serotonin to produce 20%, 50%, and 80% maximal responses were calculated when: 1) the heart was perfused with normal physiological solution, 2) the heart was perfused with solution containing d-propranolol, and 3) the heart was perfused with solution containing l-propranolol. Propranolol (25μM and 50μM) produced a surmountable blockade of the serotonin response of the heart. l-Propranolol was found to be about three times more potent than d-propranolol as a serotonergic antagonist in the heart.

Fig. A.2 The relationship between response and dose plotted semilogarithimically from an experiment made to determine the effect of propranolol (25μM) on the inotropic effect of serotonin on the perfused heart. From this graph is can be seen that both d- and l- propranolol reduce the inotropic effect of serotonin. However, l-propranolol appears to be about three times more potent than d-propranolol.


Response %
100
80
60
40
20
CONTROL
d-PROPRANOLOL
l-PROPRANOLOL
0.05
0.1
0.2
0.4
1.0
2.0
4.0
Dose ng

DISCUSSION

In the experiments on single neurones, it was observed that propranolol blocked depolarising but not hyperpolarising responses produced by serotonin or acetylcholine. These blockades were not stereospecific as the two isomers of propranolol were equipotent. The low potency and non-stereospecificity was also found in experiments in which serotonin sensitive [^{3}H]-LSD binding to snail neuronal membranes was measured (A.H.Drummond, personal communication). In these experiments, binding was inhibited by 50% by methysergide at 0.1μM, and by d-propranolol and l-propranolol at 50uM. In contrast, blockade of [^{3}H]-serotonin to rat membranes by propranolol was highly stereospecific, and was inhibited by 50% by l-propranolol at a concentration of less than 0.1μM. (Middlemiss, Blakeborough & Leather, 1977).

It therefore appeared that on snail neurones, propranolol was a non-selective, non-stereospecific serotonin antagonist. Since only depolarising responses were blocked by propranolol, it is possible that the blockade by propranolol resulted from a direct action on transmitter activated, depolarising ionic channels.

In the experiments on the perfused heart, propranolol blocked the positive inotropic effect of serotonin. Since the blockade was surmountable, it appeared that propranolol and serotonin competed for the same receptors. Stereospecificity of the blockade by propranolol, although small, also indicated that the effect of propranolol might be mediated by serotonergic receptors.

In the snail, therefore, propranolol appears to be a poor antagonist of serotonin: it was not stereospecific and its selectivity was low.

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