MORPHINE-LIKE COMPOUNDS AND
CENTRAL ACETYLCHOLINE RECEPTORS

by

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Most of these experiments were performed in collaboration with Dr A. W. Duggan. Some of the investigations with morphine and medial thalamic neurones were my own work. Some of the spinal cord studies were also performed with the additional collaboration of Dr J. Davies and Dr P. M. Headley.

JOHN G. HALL.
During the tenure of my Australian National University Postgraduate Award, the following papers have been published or submitted for publication:-

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This Thesis is dedicated to my parents and my wife, Helen.
CHAPTER I GENERAL INTRODUCTION

The various sections of this Thesis relate to investigations aimed at understanding the mechanism by which morphine-like compounds affect the central nervous system. The technique of microelectrophoresis enabled examination, at the single cell level, of the effects of opiates on the various firing patterns of central neurones. Attempts to correlate this information with the known central effects of these compounds given systemically have been made. These investigations led to studies of the interaction of opiate compounds with receptors for acetylcholine and studies on these receptors alone have also been carried out.
CHAPTER II - METHODS

A. ANIMAL PREPARATION

Cats of either sex weighing 2.5-3.5 kg were anaesthetized with pentobarbitone sodium 35 mg/kg I.P. or α-chloralose 50-60 mg/kg I.P., or were decerebrated by midbrain coagulation (Crawford & Curtis, 1966) under halothane anaesthesia. The type of anaesthetic employed depended on the particular investigation and is referred to in Results. Pentobarbitone anaesthesia was maintained with I.V. doses of a 10 mg/ml solution. Maintenance doses of chloralose were seldom needed.

Midbrain coagulation for the preparation of decerebrate animals was performed using a Wyss coagulator by passing a high frequency (500 kHz) current (35 mA for 10 secs) between adjacent pairs of coated electrodes. These were inserted stereotaxically to an intercollicular position and the whole tegmentum destroyed at this level.

In all investigations the temperature of the animals was maintained at 36-38°C by a heating pad controlled by a probe placed between the rib cage and the right scapula. Blood pressure was monitored continuously using a Statham pressure transducer (P23 Bd).

To minimize brain movements as a result of respiratory excursions and to abolish possible reflex movements from electrical stimulation of the forepaws and noxious heating of the in thalamic and substantia gelatinosa experiments footpad, animals were paralysed with gallamine triethiodide (4.0 mg/kg I.V. initially and maintained with 2.0 mg/kg every 30 mins), and artificially ventilated (38 strokes/min).
The stroke volume was adjusted to maintain the continuously monitored end tidal CO₂ level at 3.9 - 4.1%.

In thalamic experiments a bilateral pneumothorax was created as a further measure to reduce brain movements.

(a) Spinal Cord Preparation

The left external radial vein, the right common carotid artery and the trachea were cannulated. Following a midline incision of the dorsal skin over the lumbar region, the laminae of each lumbar vertebra were removed progressing in a caudal-rostral direction. In most experiments the spinal cord was sectioned at the lower thoracic level following infiltration with 1% procaine solution. The dural sac was opened dorsally and, for investigations on Renshaw cells, the seventh lumbar and first sacral ventral roots were cut and the proximal ends mounted on platinum stimulating electrodes. A platinum ball electrode placed on the dorsal surface of monitored incoming volleys and the exposed cord was immersed in paraffin oil (BP) in a pool formed by the dorsal skin flaps and the pool temperature maintained at 37°C by constant heating. A coil of silver chloride-coated silver wire covered with gauze impregnated with saline was sutured into adjacent muscle and served as a reference electrode. Small holes in the pia mater were made with fine forceps at sites appropriate for electrode entry.

(b) Thalamus Preparation

Following chloralose anaesthesia, the left external radial vein, left femoral artery and the trachea were cannulated. The animal's head was then positioned in a stereotaxic head frame and the cranium exposed by a midline
Fig. 1: Diagram showing arrangement of electronic apparatus for extracellular recording and drug administration.
incision. A small hole approximately 8 mm in diameter and centred on Horsley-Clark "AP + 7.0" co-ordinate and 15 mm to the left side of the midline suture was made with a dental drill. The exposed dura was protected from drying by covering the exposed dural surface with small pieces of polythene.

B. EXTRACELLULAR RECORDING TECHNIQUES

(a) Apparatus for Recording Extracellularly

Electrical potential changes in the extracellular environment near single neurones were recorded from the centre barrel of seven or five barrel micropipettes. This barrel contained 4 M NaCl into which was inserted a silver chloride coated silver wire which was surrounded in a medium of saline impregnated agar and encased in polythene tubing.

Conventional recording techniques were employed and a diagrammatic outline of the circuit for recording is shown in Fig. 1.

The potentials were led to a first stage cathode follower, two preamplifier stages (time constants 0.003 sec) and were displayed on an oscilloscope. The output from the cathode follower was also connected to a second amplifying system, the output of which was used to trigger a pulse generator. The size of action potentials triggering this generator was selected by using a voltage discriminator, and the derived standard pulses were counted by a ratemeter and the frequency then displayed on a pen recorder. To ensure accurate counting, both the amplified
signal and derived pulses were compared on the one oscilloscope. Synaptic responses were analysed in some cases by preparing peristimulus time histograms. This was performed with the aid of a small Ortec computer (128 bins).

(b) Location and Identification of Neurones

(i) Spinal Cord

Dorsal horn interneurones were located by four methods -

1. Spontaneous firing,
2. Firing produced by continuous ejection of an amino acid excitant from the advancing electrode,
3. Firing in response to stimulation of branches of the ipsilateral sciatic nerve.

Dorsal root volleys produced by such stimulation were monitored and the stimulus intensities necessary to activate the neurone were measured relative to that of the threshold of the largest fibres. Dorsal root volleys indicating a conduction velocity greater than 30 m/sec have been classed as derived from Aα fibres, and lesser velocities as derived from Aδ fibres (Gasser and Grundfest, 1939). No C fibres were stimulated.

4. Firing in response to "natural stimuli" - either radiant heat or deflection of hairs.

Radiant heat was produced by means of a lamp focussed to a spot approximately 3 mm in diameter on one of the digital pads. There is evidence that when skin temperature exceeds
45°C the input to the spinal cord from the irradiated area is almost exclusively from thermal nociceptors (Beck, Handwerker and Zimmermann, 1974). Skin temperature was monitored by a thermistor placed at the edge of the irradiated area and was displayed on a pen recorder together with cell firing produced by this stimulus. On these pad areas the temperature attained by 20 to 30 seconds of irradiation was between 47°C and 52°C and the latency of cell firing was 5-15 seconds. Cells firing with a shorter latency were excluded as the temperature reached at these times was unlikely to activate nociceptors.

Non-noxious stimulation, movement of hairs in the foot region, was produced by a continuous jet of air from a small polythene tube mounted on the oscillating arm of an electrical relay activated at 4 Hz by a Grass S8 stimulator. Both these types of "natural" stimulation were controlled by timing units (Venner) and the duration of their effect could be selected as required.

Renshaw cells were located by the firing in response to antidromic stimulation of the seventh lumbar and first sacral ventral roots, and by excitation in response to the electrophoretic ejection of acetylcholine.
(ii) **Medial Thalamus**

Neurones of the centre median, lateralis posterior and parafascicularis nuclei were approached at $45^\circ$ to the vertical using stereotaxic methods. Single neurones were located by responses to electrical stimulation of the forepaws (Albe Fessard and Rougeul, 1958). Current pulses, 10-15 mA, 0.1 m/sec duration and 0.2 - 1.0 Hz, were applied to pairs of fine uninsulated needles implanted in the main foot pad of each forepaw. Monitoring of the radial and ulnar nerves demonstrated that this type of stimulation activated small diameter slow-conducting fibres as well as the larger faster conducting fibres.

Difficulty was experienced in recording from single cells. In several instances although only one neurone was evoked by this method of peripheral stimulation, the ejection of an amino acid excitant activated many neurones. During spontaneous firing it was common for several neurones to fire synchronously. Cell firing evoked by electrical stimulation was also measured by preparing peristimulus time histograms.

C. **DRUG ADMINISTRATION**

(a) **Microelectrophoretic Methods**

This technique involves the ejection of ionized compounds contained in glass micropipettes into the
extracellular environment of single neurones. This procedure has been described in detail by Curtis (1964).

(i) Preparation of Micropipettes

Five or seven barrel micropipette assemblies were drawn out in a vertical pulling device and the tips broken under microscope observation to the required dimensions, most commonly 5-6 μm, by a fine glass rod mounted on a hydraulic manipulator (De Fonbrune, Paris). Aqueous solutions of the salts of the compounds to be ejected electrophoretically were centrifuged (1600 g) for 10 minutes and pipetted into individual barrels of the micropipette assembly. Following centrifugation (1600 g, 13 mins) which propelled the solutions to the tip, the resistance of each barrel was measured and the micropipettes stored in a bell jar until experimentation. Electrodes were used within 3 days of preparation.

(ii) Solutions

The solutions used in the investigations of this thesis, together with concentrations (and pH if adjusted) are listed in Table I.

(iii) Some Considerations On The Use of Microelectrophoresis

The amount of a compound ejected electrophoretically is a function of the current that is passed between the micropipette barrel and the nervous tissue. This "ejecting current" was supplied by the polarizer circuit shown in Figure 1 and current magnitude could be varied as required. A retaining voltage (commonly 0.5 V) of opposite polarity to that
<table>
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<th>Compound</th>
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<th>pH (if adjusted)</th>
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<tr>
<td>Acetylcholine Cl</td>
<td>0.5M</td>
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<tr>
<td>Acetyl-β-Methylcholine Cl</td>
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<tr>
<td>Atropine SO\textsubscript{4}</td>
<td>10mM in 0.1M NaCl</td>
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<tr>
<td>α Bungarotoxin</td>
<td>1.5 x 10\textsuperscript{-2} g/ml</td>
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<tr>
<td>Dextrorphan tartrate</td>
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<td>Dihydro-β-erythroidine</td>
<td>10mM in 0.1M NaCl</td>
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<tr>
<td>γ-amino butyric acid (HCl)</td>
<td>0.5M</td>
<td>3.0</td>
</tr>
<tr>
<td>Glycine (HCl)</td>
<td>0.5M</td>
<td>3.0</td>
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<tr>
<td>Levallophan tartrate</td>
<td>0.1M</td>
<td></td>
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<tr>
<td>Levorphanol tartrate</td>
<td>0.1M &amp; 25mM in 0.1M NaCl</td>
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<tr>
<td>Morphine SO\textsubscript{4}</td>
<td>0.07M</td>
<td></td>
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<tr>
<td>Nalorphine HBr</td>
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<td></td>
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<tr>
<td>Naloxone HCl</td>
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<td></td>
</tr>
<tr>
<td>Nicotine HCl</td>
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<td>7.5</td>
</tr>
<tr>
<td>Oxymorphine HCl</td>
<td>0.1M</td>
<td></td>
</tr>
<tr>
<td>Physostigmine salicilate</td>
<td>10mM in 0.1M NaCl</td>
<td></td>
</tr>
<tr>
<td>Sodium L-aspartic acid</td>
<td>0.5M</td>
<td>6.8</td>
</tr>
<tr>
<td>Sodium L-glutamic acid</td>
<td>0.5M</td>
<td>7.2</td>
</tr>
<tr>
<td>Sodium DL-homocysteic acid</td>
<td>0.2M</td>
<td>7</td>
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for ejecting the compound was applied continuously to each drug containing barrel.

While the concentration of an ejected compound in the extracellular environment cannot be accurately determined, it is possible to compare, on the basis of ejecting currents, the relative potency of one compound on different types of neurones or of several compounds on one type of neurone (Duggan, 1974).

A considerable proportion of the investigations described in this thesis is concerned with reduction by a locally or intravenously administered compound of neuronal firing in response to a chemical, physiological or artificial stimulus. It must be demonstrated by the use of adequate controls that such an effect, particularly when observed over an extended period of time, was a direct consequence of the compound administered and not of variation of physiological conditions. Similarly recovery must, when possible, be observed. The changes in blood pressure and respiration consequent upon intravenous administration of morphine-like compounds are well known, and any resultant tissue movement may well lead to alterations in extracellular recording parameters caused by alteration in pipette to cell distances. The artifactual changes produced by these effects when electrophoretic ejection is occurring are important when concentrations at receptor sites are considered. For these reasons it was essential to observe alternately the effect
believed susceptible to the administered compound concommitantly with an effect thought not to be susceptible. For example, to show that the antagonism by electrophoretically administered naloxone of the excitation of a Renshaw cell induced by morphine was a true effect and not due to increasing pipette-cell distances (a situation that would "dilute" the effect of morphine anyway), responses to an amino acid excitant were elicited alternately with those of morphine. Similarly, the effects of opiates on the responses of dorsal horn interneurones to noxious stimulation were always determined in the presence of responses to "non-noxious" stimulation. When testing for the anticholinergic actions of the snake neurotoxins, an amino acid excitant was alternately ejected with acetylcholine.

The use of a retaining voltage results in a progressive lowering of drug concentrations in the tip of a micropipette. Compounds were therefore administered for fixed times and at fixed intervals and responses elicited were only considered control responses when they were observed to be reproducible on successive ejections.

(iv) Two-electrode Experiments

In an endeavour to reveal effects of electrophoretically ejected opiates on the responses of dorsal horn interneurones to noxious stimulation of the footpads, an attempt was made to administer drugs to the dendritic regions, as distinct from the soma, of single neurones. This involved the
apposition under microscopic observation of the tips of a five and a seven barrel micropipette just above the dorsal surface of the spinal cord, and noting the respective manipulator co-ordinate readings. The seven barrel micropipette, containing drugs for influencing the dendritic processes, was advanced vertically into the cord, until neuronal activity as a result of an ejected amino acid excitant was first encountered. These neurones were usually 0.8-1.0 mm below the dorsal surface of the cord and deposits of pontamine sky blue subsequently showed them to be located in Rexed lamina I (Rexed, 1954). The micropipette was then advanced 50-200 µm ventrally so as to lie in the region corresponding to Rexed laminae II or III, the substantia gelatinosa. As well as containing small neurones, this region is considered to contain the dendrites of more ventrally situated neurones (Szentágothai, 1964). It was from these lamina IV and V neurones that single neurone recording was achieved with the five barrel micropipette. The five barrel micropipette, the precise position of which relative to the other was known, was then advanced into the cord at 18° to the vertical, passing 200-350 µm beneath the tip of the seven barrel micropipette. When approaching this position, neurones were located by firing evoked chemically or synaptically (See Fig. 23). Neurones were accepted for testing if they were located within 600 µm of the substantia gelatinosa pipette and if they were excited by both noxious and non-noxious
stimulation.

(b) **Administration By Pressure**

(i) **Pharmacology of Snake Toxins**

When it became apparent that Renshaw cell responses were not influenced by snake toxins administered electrophoretically, administration by pressure was used in case the lack of response was due to failure of electrophoretic administration. Cobra neurotoxin and α-bungarotoxin was supplied by Professor C.Y. Lee having been purified from the whole venoms of *Naja naja atra* and *Bungarus multicinctus* according to the procedure described previously (Lee et al., 1968; Lee et al., 1972). The several methods of administering the compounds by pressure are summarized below –

1. α-Bungarotoxin (1.5 x 10^{-2} g/ml solution)
   - By applying air pressure of up to 300 mm Hg to the toxin containing barrels of seven barrel micropipettes of tip diameters 5-8 μm. The pressure of air in the system was measured with a mercury manometer.

2. Cobra Neurotoxin (0.05, 0.5 or 1.5 x 10^{-2}) g/ml solution).
   - By applying a pressure of up to 300 mm Hg to a single toxin-containing micropipette of tip diameter 8-15 μm cemented to a 5-barrel micropipette assembly which projected 30-50 μm beyond the tip of the toxin containing pipette (A "parallel" electrode, Curtis, 1968).
- By applying a pressure of up to 300 mm Hg to the toxin containing barrel of 5-barrel micropipettes with relatively large tips (7-10 μm).

- By ejecting relatively large amounts of toxin from a single micropipette of tip diameter 50-80 μm at distances of 1-20 mm from Renshaw cells from which records were being obtained using a separate 5-barrel micropipette. From the changes in the level of solution within the pipette, the volumes administered were estimated to be between 10 and 30 μl.

Unlike ejection by electrophoresis where the current provides a measure of the amount of substance being administered from the tip, ejection by pressure cannot be quantified. In determining whether toxin was being ejected the following observations could be made:

- A reversible change in action potential amplitude on applying pressure to the toxin containing barrel.

- An increase in spontaneous firing immediately following ejection, presumably a result of mechanical disturbances in the tissue caused by solution ejection.

- Changes in cell firing following pressure applied to an adjacent barrel of the micropipette assembly. This was either
excitation when an amino acid excitant was ejected or a reduction in the response to acetylcholine or to a ventral root stimulus following application of pressure to a barrel containing dihydro-β-erythroidine. These controls, while not being strictly adequate for ejection from the toxin containing barrel, gave an indication of the extent to which tissue debris may have been occluding the whole micropipette assembly (see Andersen & Curtis, 1964).

Microscopic observation of ejection into oil or of streaming of a talc suspension in the vicinity of the electrode tip when pressure was applied to the toxin-containing barrel both before and after insertion into the spinal cord.

The purified toxins were iodinated by Dr. I. Hendry of this Department according to the method of Berg et al., (1972) and were ejected in the vicinity of Renshaw cells by pressures of up to 300 mm Hg from single micropipettes of tip diameter 15-25 μm. The precise location of Renshaw cells was determined by prior penetration of the cord with a micropipette while stimulating ventral roots. The amount and rate of ejection was controlled by directly observing the fall in the level of the toxin within the pipette. This had been
approximately calibrated by comparison with levels in similar pipettes containing 0.5 and 1.0 μl of water. At each injection site, between 0.06 and 0.1 μl of neurotoxin was expelled. Partial blockage of the tip of the micropipette by tissue was common as evidenced by variations in the pressure necessary to expel this volume over a period of 1-3 minutes. As well as injections adjacent to Renshaw cells, neurotoxin was also deposited in the dorsal horn during withdrawal of the electrode. A series of spaced (3-7 mm) injections were made sufficient to avoid cross-contamination for diffusion times up to 7 hours.

(c) Intravenous Administration

Solutions administered intravenously were:

- Morphine sulphate 5 or 10 mg ml\(^{-1}\)
- Naloxone hydrochloride 0.5 or 1.0 mg ml\(^{-1}\)
- Levorphanol tartrate 1.0 mg ml\(^{-1}\)
- Levallorphan tartrate 0.5 or 1.0 mg ml\(^{-1}\)

All solutions were made up in .165 M NaCl.

Because of the circulatory effects of morphine, this compound was administered in a series of gradually increasing doses (Schmitt and Livingston, 1933). An initial dose of 0.5-1.0 mg kg\(^{-1}\) was usually given and succeeding doses, at 5 to 10 minute intervals. All intravenous compounds were administered into the left external radial vein.
D. **HISTOLOGY**

(a) **Dye Marking Techniques**

In many experiments the site of extracellular recording of a particular cell was marked by ejecting a dye electrophoretically. The dye solutions used were Pontamine Sky Blue, 2% in 0.5 M sodium acetate (Hellon, 1970), and Acid Fast Green, saturated solution in 2 M NaCl (Thomas and Wilson, 1965). Both dyes were ejected as anions, Pontamine Sky Blue at 4 μA for 5 mins and Acid Fast Green at 5 μA for 10 mins.

At the conclusion of each thalamic experiment a mounted scalpel blade replaced the micropipette assembly in its holder and was lowered into the exposed cerebrum at distances of about 3 mm both caudal and rostral to the site of the electrode track. Once the entire brain was removed, these incisions enabled removal of a block of tissue containing the appropriate area of thalamus and permitted the cutting of sections in approximate Horsley Clarke frontal planes. This was trimmed and fixed in 10% formal saline for 3 days. The fixed block was then mounted on a freezing microtome and coronal sections 50 μm thick were cut and examined microscopically for the small dye spots. Two pontamine sky blue spots were deposited, one at the site of recording and another 3.0 mm superficial and it was common to find both spots within 3 or 4 sections of each other. The sections were blotted flat onto a glass slide and kept moist with distilled water. A photographic enlargement of the unstained section was then made and onto it were marked
the positions of the dye spots.

At the conclusion of spinal cord experiments, a centimetre length of cord was removed containing an Acid Fast Green spot at the site of extracellular recording in the vicinity of lamina IV or V and a Pontamine Sky Blue spot in the substantia gelatinosa region. The tissue was then fixed, sectioned and photographed as for thalamic tissue.

(b) Autoradiography

As this technique requires that the fixation of tissue be more thorough than that obtained by immersing the tissue block in formal saline, the anaesthetised animals, injected intraspinally with $^{125}$I-labelled snake neurotoxins, were killed by bleeding from an incision in the right atrium immediately following the start of the perfusion of the spinal cord with 320 mOsM Phosphate buffer, pH 7.4 (Karlsson and Schultz, 1965) containing 4% paraformaldehyde and 1 mM CaCl$_2$. This was via a cannula in the thoracic descending aorta, both common iliac arteries being ligated. The fixed cord was divided into blocks of 4.5 to 7 mm, each being approximately centred on an injection site. Light microscopy autoradiography was performed by Dr. I. Hendry and each tissue block was embedded in paraffin and serial sections of 5 μm were prepared.
CHAPTER III - STUDIES WITH MORPHINE-LIKE COMPOUNDS

A. EFFECTS OF OPIATE AGONISTS AND ANTAGONISTS ADMINISTERED NEAR THE SOMA OF SPINAL NEURONES

Introduction

Chemical synapses are a possible site of action for centrally active compounds. Thus many pharmacological, biochemical and anatomical investigations have attempted to relate the central effect of morphine-like compounds (opiates) to effects on specific neurotransmitter systems.

Substantial evidence has been obtained for the existence of discrete "opiate receptors", the bulk of this information being derived from in vitro studies of the binding of radioactively-labelled opiate compounds to homogenates of brain tissue (Goldstein, Lowney and Pal, 1971; see Snyder, Pasternak and Pert, 1975). One component of this binding, termed "stereospecific", is believed relevant to the interactions of systemic opiates. Stereospecific binding in these experiments is defined as the difference in displacement of a labelled opiate compound by high concentration of an active opiate and that by an inactive isomer (see Goldstein et al. 1971). Several lines of evidence substantiate the relevance of these receptors; opiate compounds compete with a labelled compound in a manner related to their relative pharmacological potencies (Pert and Snyder, 1973b; Wilson, Rogers, Pert and Snyder, 1975) and, of a wide range of compounds tested, only those classed as having opiate activity were able to compete for binding sites to a significant extent (Pert & Snyder, 1973a).

Although subcellular localization of these "opiate
receptors" has shown them to be associated with synaptic membranes (Pert, Snowman & Snyder, 1974), the precise relationship of these receptors to presynaptic or postsynaptic structures has yet to be determined. Additional studies (Snyder, Pasternak & Pert, 1975) have suggested that a consistent relationship between the levels of a particular synaptic transmitter and the amount of opiate receptor binding does not exist and that selective lesions aimed at reducing the levels, predominantly of one probable transmitter, does not alter the magnitude of binding, despite large decrements in the levels of that transmitter (Kuhar, Pert & Snyder, 1973). Such studies would indicate that the opiate receptor is not a unique component of axons or nerve terminals of any one of the transmitter systems.

An ever increasing body of information suggests that morphine-like compounds have actions at central receptors for acetylcholine. This work was prompted initially by the finding that in the guinea pig ileum preparation, the reduction by morphine of stimulation-induced contractions was accompanied by decreased acetylcholine release (Paton, 1957). There is evidence that morphine-like compounds increase the steady-state levels of acetylcholine in the central as well as the peripheral nervous systems. (Crossland & Slater, 1968; Giarman & Pepeu, 1962; Morris, 1961).

The reduction by morphine of the release of acetylcholine from brain tissue into cerebrospinal fluid (Beleslin & Polak, 1965; Crossland & Slater, 1968; Jhamandas, Phillis & Pinsky, 1971; Sharkawi & Shulman, 1969) suggests that the increased acetylcholine levels in the tissue are a result of interference with its release. An increase in the synthesis of acetylcholine
following morphine has not been demonstrated (De La Lande and Bentley, 1955; Morris, 1961) and neither has any inhibition of brain cholinesterase activity been detected (Ettinger & Gero, 1966; Dewey, Harris, Howes, Kennedy, Nuite & Hayhurst, 1969). Furthermore, the depletion of cerebral acetylcholine by hemicholinium was prevented by doses of morphine too low to enhance acetylcholine levels themselves, an effect antagonised by nalorphine and naloxone (Domino & Wilson, 1973). Clouet & Williams (1974), employing in vitro methods, have found that morphine and some related compounds reduced the acetylcholine release from synaptosomal preparations of rat brain in a manner generally related to their relative antinociceptive potencies.

Phillis, Mullin & Pinsky (1973) have recently shown that morphine enhances acetylcholine release from the cerebral cortex in unanaesthetized cats. In similar experiments on mid-pontine-pretrigeminal cats, Yaksh & Yamamura (1973) showed that morphine blocked acetylcholine release from the artificially perfused caudate nucleus, an effect antagonized by naloxone.

There are other lines of evidence that morphine and other opiate analgesics have effects at receptors for acetylcholine. Bhargava & Way (1972) have shown that di-isopropylfluorophosphate (DFP), a potent acetylcholinesterase inhibitor, strongly enhances morphine analgesia. Other cholinesterase inhibitors such as physostigmine and neostigmine also potentiate analgesia (Slaughter & Gross, 1940; Harris, Dewey, Howes, Kennedy & Pars, 1969; Saxena & Gupta, 1957; Szerb, 1957). Oxotremorine, a muscarinic agonist (Cho, Haslett & Jenden, 1962) is more effective than morphine in producing inhibition of tail-flick in mice and was only weakly antagonized by naloxone (Harris et al., 1969; Ireson, 1970;

These latter findings suggest that opiates increase activity at cholinergic synaptic regions while inhibition of release was the mechanism proposed by the literature cited earlier.

Investigations at sites of cholinergic transmission in the peripheral nervous system have shown that opiates have effects on synaptic processes. Morphine has a depressant action on neuromuscular transmission (Soteropoulos & Standaert, 1973). Pinsky & Frederickson (1971) and Frederickson & Pinsky (1971) demonstrated that the release of acetylcholine in the rat phrenic nerve-diaphragm and frog sartorius preparations was decreased by morphine. No antagonism of these effects or correlation with analgesic potency was observed by Bell & Rees (1974) however, and it can reasonably be concluded that effects at the neuromuscular junction are not related to effects at stereospecific sites.

Contractions of the electrically stimulated guinea pig ileum strip are blocked by morphine (Schaumann, 1957), and Paton (1957) has further demonstrated that this effect is closely related to a reduction of acetylcholine released into the organ bath following each stimulus. This preparation has been widely used to demonstrate antagonism of morphine effects by antagonists, the production of tolerance and the close correspondence of the potencies of compounds with their analgesic potencies (Kosterlitz & Watt, 1968; Gyang & Kosterlitz, 1969). The complex organization of the mural plexus of nerve cells within the ileum however makes these observations difficult to interpret and difficult to relate to effects on single central neurones. In cardiac tissue, Kennedy & West (1967) have also proposed an inhibition of
acetylcholine release by morphine.

Studies of the phenomenon of tolerance to and dependence upon opiates have also implicated a role for cholinergic mechanisms in these processes (Paton, 1963). Several reports have found that cholinergic agonists inhibited, while antagonists exacerbated the withdrawal symptoms of dependent animals (Grumbach, 1969; Brase, Tseng, Loh & Way, 1974, but see Jhamandas & Dickinson, 1973). A postsynaptic sensitizing action of morphine has been suggested, from studies in the peripheral nervous system, as the basis of these phenomena (Pollock, Muir & MacDonald, 1972; Muir & Pollock, 1973; but see Haycock & Rees, 1973).

In summary then, the literature concerning the interaction of morphine-like compounds with cholinergic synaptic processes does not suggest one particular mode of action, and although an inhibition of acetylcholine release is held to be an important effect, at least in the peripheral nervous system, there are many reports casting doubt on whether this mechanism is a sufficient explanation for all of morphine's actions.

Using microelectrophoretic techniques, cholinoceptive neurones have been found in many regions of the mammalian central nervous system (for reviews see McLennan, 1970; Phillis, 1970; Krnjević, 1974) but as yet there is good evidence for cholinergic transmission at only one site - the synapses between motoneurone axon collaterals and Renshaw cells (Eccles, Fatt & Koketsu, 1954; Curtis & Eccles, 1958). Felpel, Sinclair & Yim (1968; 1970) provided the first published account of the effects of morphine on Renshaw cell activity. In an earlier study, Kruglov (1964) had observed that intravenous morphine 5-10 mg kg$^{-1}$, reduced recurrent but not direct inhibition of a monosynaptic spinal
reflex, an effect which was attributed to impaired activity at the axon collateral-Renshaw cell synapses. Felpel et al. (1968; 1970), however found that Renshaw cell discharges evoked by ventral root stimulation or by intravenous nicotine were not significantly depressed by morphine, despite the reduction in recurrent inhibition and concluded that the effect of morphine could not be ascribed to an action at this site.

Subsequently, Curtis & Duggan (1969), investigating the nature of the transmitter mediating inhibition at motoneurones, found that intravenous doses of morphine given in divided doses to a total of 50 mg kg$^{-1}$ reduced direct as well as recurrent inhibition in a roughly parallel fashion. By demonstrating, in addition, morphine's antagonism of the inhibitory actions of glycine, when both substances were administered electrophoretically, they suggested that if a glycine-like transmitter mediated motoneurone inhibition then this might explain the reduction of both types of reflex by morphine.

In a similar, but later investigation (Duggan & Curtis, 1972), the same workers examined the effects of electrophoretically and intravenously administered morphine on the latencies of Renshaw cell discharges following ventral root stimulation. They found that electrophoretically administered morphine increased both the latency and the number of individual action potentials following a ventral root stimulus. This latter effect, which was also an effect produced by acetylcholine and DL-homocysteate, they interpreted as a postsynaptic effect of the alkaloid. The increase in latency of each action potential was likened to the effect seen with
hemicholinium (Quastel & Curtis, 1965; Lodge, Curtis & Craig, 1975) and was therefore suggested to be a presynaptic effect, presumably resulting from an interference by morphine of acetylcholine release. Similar results have been obtained in the rat (Lodge, Headley, Duggan & Biscoe, 1974).

The investigations described here are an endeavour to elucidate further the interaction of opiate compounds with central acetylcholine receptors. The cholinoceptive Renshaw cell has been the site for this study and the effects observed with morphine-like compounds have been contrasted with those at non-cholinoceptive neurones of the spinal cord. It must at the outset be emphasized that Renshaw cells were employed in these investigations because of the well established cholinergic nature of the transmission at this synapse and that a role for this site in the mechanisms of morphine analgesia is unlikely.

In the initial studies experiments were performed on unparalysed pentobarbitone-anaesthetized animals.
RESULTS

(i) STUDIES WITH CHOLINOCEPTIVE NEURONES

(a) Morphine Administered Electrophoretically

Morphine ejected electrophoretically was tested on 90 Renshaw cells. Three dose-dependent effects were observed: - excitation, antagonism of the depression of cell firing by glycine, and an increase in the latency of the initial action potentials of the synaptic response evoked by a submaximal ventral root stimulus.

Morphine excited 70 of these 90 Renshaw cells when ejected with currents of 25-80 nA. The excitation was slower in onset than that produced by acetylcholine, but recovery was usually rapid on termination of the ejecting current. On the basis of ejecting currents required to produce equal increases in neuronal firing, the potency of morphine was the same order as that of L-glutamate and L-aspartate, but approximately one fifth to one tenth that of acetylcholine. Excitation of a Renshaw cell by morphine, L-aspartate and acetylcholine is illustrated in Figure 2.

The sensitivity of Renshaw cells to morphine was related to their sensitivity to acetylcholine, and the significance of this correlation is shown in Table II. With cells relatively insensitive to acetylcholine, the ejection of morphine with large currents resulted in firing in bursts with little
Fig. 2: Excitation of Renshaw cells by morphine. Each trace is a ratemeter record of cell firing. Ordinate: spikes per second. Abscissa: time in minutes. The times of ejection of substances electrophoretically are indicated by symbols above each tracing. A. Excitation by morphine (MORPH), acetylcholine (ACH) and L-aspartate (L-ASP). B. Enhancement of excitation by acetylcholine but not DL-homocysteate (DLH) during ejection of morphine. C. Enhancement of the excitatory actions of both acetylcholine and L-aspartate during ejection of morphine.
# Table II

<table>
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<th>Acetylcholine Response</th>
<th>Ejecting current to attain 20 spikes/sec.</th>
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<td>Morphine Response</td>
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<td>&gt;50 nA</td>
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<td>21</td>
<td>18</td>
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<td>&lt;50 nA</td>
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<td>1</td>
<td>19</td>
</tr>
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Ejecting current to attain 20 spikes/sec.
increase in mean firing rate.

Excitation by morphine was also manifested by an increase in the total number of action potentials evoked by a ventral root stimulus and enhancement of the actions of other excitants. In the rat, morphine has been reported to enhance the action of acetylcholine with little effect on that of other excitant amino acids (Lodge et al., 1974). This was observed on only 3 of 9 neurones in the present investigations (Fig. 2B), the more usual finding being enhancement of the action of all excitants tested (Fig. 2C).

The previously described actions of electrophoretically administered morphine on ventral root evoked firing of Renshaw cells (Duggan & Curtis, 1972) were confirmed in the present experiments and are discussed below. Another effect previously reported, the reduction by morphine of the depressant action of glycine (Curtis & Duggan, 1969) was also confirmed, and several investigations were carried out to determine which of these effects of morphine occurred with the lowest ejecting current. There was no significant difference in the currents of morphine required to produce just detectable excitation and just detectable reduction of glycine depression.

(b) Naloxone and Nalorphine Administered Electrophoretically

These compounds are n-allyl derivatives of the oxymorphone and morphine molecules which endows them with opiate antagonist properties. Their structures together with that of morphine and oxymorphone are
Fig. 3: The chemical structures of morphine, nalorphine, oxymorphone and naloxone.
Fig. 4: The effects of naloxone and morphine on the latencies of the initial action potentials of a Renshaw cell following a ventral root stimulus. Each histogram represents the sum of 128 responses analysed in 100 μsec intervals. Abscissae: time in msec after the ventral root stimulus. A. Controls. B. 2½ minutes from the start of naloxone (30 nA) ejection. C. 5 minutes from the start of naloxone (30 nA) ejection and 1½ minutes from the start of added ejection of morphine (30 nA). D. 4½ minutes from the cessation of ejection of morphine and naloxone. The reduction in action potential amplitude during ejection of naloxone and morphine reduced the total counts of the second and third action potentials as these were superimposed on a positive field potential.
shown in Fig. 3.

These substances were tested both for effects on cell firing *per se* and for possible antagonism of the effects of morphine.

Naloxone ejected with currents of up to 30 nA had little effect on cell firing. Ejected with higher currents, up to 60 nA, and for times used to demonstrate excitation by morphine (up to 2 minutes), naloxone excited 2 of 19 Renshaw cells. Bursts of action potentials and a decrease in amplitude similar to those produced by high currents of morphine could be observed at these excessive currents. When ejected for longer periods, during tests for antagonism of morphine's effects, excitation by naloxone in the form of enhancement of either spontaneous firing or the action of an excitant amino acid, was observed with 9 of 19 neurones. The firing rate attained under these conditions was always less than fifty per cent of that produced by similar currents of morphine ejected for much shorter times.

Naloxone (20-50 nA) was morphine-like in that it increased the latency of action potentials evoked by a submaximal ventral root stimulus on 6 neurones, the results from one being shown in Fig. 4. Also illustrated is that morphine, ejected subsequently to naloxone, further increased cell latencies, the effects of the two substances being additive rather than antagonistic. Tests in which morphine was ejected prior to naloxone failed to detect antagonism between these two compounds.
Fig. 5: Failure of naloxone to antagonize the reduction by morphine of glycine depression of the firing of a Renshaw cell. Spontaneous firing was depressed by the alternated ejection of glycine and γ-aminobutyric acid (GABA.)

A. Naloxone ejection commenced after that of morphine.
B. On the same cell naloxone ejection commenced prior to that of morphine.
Fig. 6: Antagonism by naloxone of excitation of a Renshaw cell by morphine and acetylcholine. A. Reduction of excitation by morphine and acetylcholine (ACH) during ejection of naloxone. B. Reduction of excitation by morphine with little effect on that of DL-homocysteate (DLH) during ejection of naloxone.
Naloxone (10-60 nA) had no effect on the depression of cell firing by glycine and GABA and did not antagonize the reduction of the action of glycine by morphine on all seven cells tested. This is illustrated in two ways in Figure 5. When naloxone was ejected during the administration of morphine the depressant action of glycine, previously blocked, did not alter (Fig. 5A) whilst Figure 5B shows that on the same Renshaw cell, a concentration of naloxone without effect on the actions of glycine or GABA did not prevent the antagonism of glycine by subsequent morphine. Higher currents of naloxone relative to morphine were difficult to test and these substances were additive in reducing action potential amplitudes.

Naloxone ejected with currents of 10-30 nA antagonized excitation by morphine and by acetylcholine but not that by an amino acid. This occurred with 15 of 18 Renshaw cells tested and results from one are shown in Figure 6. Concentrations of naloxone adequate to reduce the action of morphine were, on 9 occasions, weakly excitatory and Figure 6A also illustrates this.

An attempt was made to determine if naloxone was more potent as a morphine antagonist than as an acetylcholine antagonist. Two findings suggested that this was not so: - firstly a parallel reduction in the excitation produced by morphine and by acetylcholine, and secondly, when naloxone was tested against enhancement of the excitatory response to
Fig. 7: Antagonism by nalorphine of excitation of a Renshaw cell by morphine and acetylcholine. A. Reduction of excitation by morphine with little effect on that of L-glutamate (L-GLUT) during ejection of nalorphine. B. Reduction of excitation by acetylcholine (ACH) with little effect on that of DL-homocysteate (DLH) during ejection of nalorphine.
acetylcholine and an amino acid by morphine, the action of acetylcholine was reduced more than that of the amino acid.

As discussed previously, a postsynaptic sensitizing action by morphine of the response of the colon to acetylcholine has been proposed (Pollock et al., 1972; Muir & Pollock, 1973). It is possible then that the reduction of the action of acetylcholine by naloxone was antagonism of the action of morphine on cells that had been sensitized to acetylcholine by the leakage of small amounts of morphine from micropipettes. Four experiments were therefore performed using micropipettes, none of the barrels of which contained morphine, but naloxone still reduced the action of acetylcholine on Renshaw cells.

Nalorphine was similar to naloxone in reducing excitation by morphine and by acetylcholine with little or no effect on that by an amino acid. This was observed on 5 of 7 neurones tested and results from one are illustrated in Figure 7. A weak excitatory action by nalorphine, similar to that produced by naloxone could be detected after prolonged administration. Nalorphine was not adequately tested for antagonism of the suppression by morphine of the inhibitory action of glycine, but was found to be morphine-like in prolonging, on the one neurone tested, the latency of action potentials evoked by a ventral root stimulus.

(c) The Pharmacology of Morphine Excitation

Renshaw cell cholinergic receptors have been
Fig. 8: Effects of naloxone on excitation of a Renshaw cell by acetylcholine, acetyl-β-methylcholine and nicotine. A. Reduction by naloxone of the excitant effect of acetylcholine (ACH) but not that of acetyl-β-methylcholine (Ac-β-METH) and DL-homocysteate (DLH) on a Renshaw cell. Naloxone (10 nA) was ejected for a total of 7½ minutes not all of which is illustrated. B. Reduction by naloxone of excitation of a Renshaw cell by acetylcholine and nicotine but not that by DL-homocysteate. These records were obtained from the same cell as those of A. Naloxone (10 nA) was ejected for a total of 9½ minutes not all of which is illustrated.
shown by Curtis & Ryall (1966) to be of both nicotinic and muscarinic types. It was therefore of interest to determine in more detail the pharmacology of excitation by morphine as it became apparent that this activity was exerted at the same or similar receptors as those for acetylcholine. Experiments were carried out to ascertain the effects of naloxone on the excitation of Renshaw cells by nicotinic and muscarinic agonists (nicotine and acetyl-β-methylcholine respectively), and of nicotinic and muscarinic antagonists (dihydro-β-erythroidine and atropine respectively) on excitation by morphine.

In these tests, naloxone consistently reduced the excitation by acetylcholine (15 cells) and nicotine (8 cells) but had no effect or enhanced that produced by acetyl-β-methylcholine (9 cells) and DLH (9 cells). The results obtained from one Renshaw cell are shown in Figure 8.

Dihydro-β-erythroidine (DHβE) ejected with small currents (4-10 nA), reduced in parallel the excitation of 4 of 5 neurones by acetylcholine and morphine. With one neurone, acetylcholine excitation was reduced while that of morphine was unaffected by DHβE. Atropine reduced morphine and acetylcholine responses in parallel on 4 neurones although relatively high currents of atropine (25-40 nA) were required and the effects were small compared with those of DHβE. Neither of these two antagonists affected the excitatory responses of amino acids. Figure 9 illustrates the actions of
Fig. 9: Effects of dihydro-ß-erythroidine and atropine on excitation of a Renshaw cell by morphine, acetylcholine and DL-homocysteate. A. Reduction by dihydro-ß-erythroidine (DHßE) of the excitant effect of morphine and acetylcholine (ACH) but not that of DL-homocysteate (DLH) on a Renshaw cell. Not all the recovery period is illustrated the gap in record representing 6 minutes. B. Slight reduction of the effects of acetylcholine and of morphine but not that of DL-homocysteate during the ejection of atropine with relatively large currents. Atropine was ejected with a current of 10 nA for 3 minutes, 20 nA for 3 minutes, 30 nA for 3 minutes and 40 nA for 1½ minutes. Not all of the responses during this total period of ejection are illustrated. The recovery responses were observed two minutes after the cessation of atropine ejection.
DHßE and atropine on the responses of a Renshaw cell to morphine, acetylcholine and DLH.

(d) Intravenously Administered Morphine and Naloxone

Results concerning the effects of intravenous morphine and naloxone on the firing of Renshaw cells in response to a submaximal ventral root stimulus were obtained from 10 animals.

Morphine was administered in a series of gradually increasing doses as described in Chapter II. Whilst the hypotension and pulse pressure fluctuation were minimized by this measure (see Fig. 20), severe respiratory depression was still present with the doses employed as elevated levels of end tidal CO₂ could be observed. Naloxone readily reversed both the respiratory and circulatory effects of morphine (see Fig. 20).

Peri-stimulus time histograms of ventral root responses of 8 Renshaw cells indicated that morphine in doses of 1-4 mg kg⁻¹ enhanced the response in four experiments (2 with the spinal cord sectioned) and had no effect in four others.

The enhancement of ventral root firing following intravenous morphine was reversed by naloxone in all of three experiments and results from one are presented in Figure 10.

Naloxone given alone in doses of 0.1 - 0.5 mg kg⁻¹ had no significant effects on blood pressure or on the firing of Renshaw cells by ventral root volleys in 4 experiments. In a fifth animal such firing was depressed by about 25% following naloxone (0.3 mg kg⁻¹).
Fig. 10: Enhancement of ventral root evoked firing of a Renshaw cell by intravenous morphine and reversal of this effect by subsequent naloxone. Each histogram represents the sum of 128 responses analysed in 200 μs intervals. Abscissa: time in ms after the ventral root stimulus. Morphine was administered intravenously in three divided doses of 1 mg/kg at 2½ minute intervals, the middle record was computed 1 minute after the last dose of morphine. Naloxone 0.1 mg/kg was given intravenously 3 minutes after the last dose of morphine and the lower record computed 1½ minutes subsequently.
Higher doses of naloxone (2-3 mg kg⁻¹) were given to four pentobarbitone-anaesthetized spinal animals. These doses had no significant effects on blood pressure but in three animals limb movements occurred. In three of these experiments, the total number of action potentials evoked by a submaximal ventral root stimulus was decreased 30-60% following intravenous naloxone. In a fourth, an increase of 50% was observed in the number of evoked action potentials, but "arousal" with naloxone was marked and spontaneous firing also increased by approximately 50%.

A specific effect of high doses of naloxone (2.7 mg/kg) on cholinergic synaptic activation of Renshaw cells from ventral roots, by comparison to activation from the dorsal roots, could be demonstrated in only 2 of 7 experiments. In 3 cases both types of activation were depressed, and in one other the dorsal root firing was increased with no effect on that of the ventral root. In the remaining experiment naloxone was without effect on either type of activation.

(e) **Studies with Morphinan Compounds**

1. **Administered Electrophoretically**

As a further test of the specificity of the observed effects of morphine at nicotinic receptors on Renshaw cells, investigations were carried out using three morphinan compounds; levorphanol an active opiate analgesic (Fromherz, 1951), dextrorphan an inactive isomer (Fromherz
Fig. 11: The chemical structures of levorphanol, levallorphan and dextorphran.
and levallorphan an opiate antagonist (Fromherz & Pelmont, 1952). The structures of these compounds are shown in Figure 11.

Ejected from an 0.1 M solution, levorphanol (10-40 nA) excited 5 of 14 Renshaw cells. Ejected from a 25 mM solution in 100 mM NaCl levorphanol (20-100 nA) excited 18 of 24 Renshaw cells. Cells fired by an ejecting current of acetylcholine of less than 10 nA were usually also fired by levorphanol, whereas with cells less sensitive to acetylcholine, high currents of levorphanol, like those of morphine, tended to produce bursts of action potentials of diminished amplitude with little increase in mean firing rate.

In an attempt to compare the potency of levorphanol with that of morphine both substances were administered (levorphanol in NaCl) with similar currents to 8 Renshaw cells. In these tests the currents of morphine and of levorphanol to produce equal increases in cell firing were not significantly different and, as only a proportion of the ejecting current was carried by levorphanol ions, it is probable that levorphanol was more potent as an excitant than morphine. Figure 12A illustrates the responses of one cell to acetylcholine, morphine and levorphanol and the lack of excitation by dextrorphan (see below).

The excitation by levorphanol of 8 of 9 Renshaw cells was reduced or abolished by
Fig. 12: Excitation of a Renshaw cell by levorphanol and antagonism of this effect by naloxone. A. The responses to the ejection of acetylcholine (ACH 10 nA), levorphanol (LOR 40 nA), dextrorphan (DEXTR 40 nA) and morphine (MORPH 40 nA). B. Reduction of the excitation by levorphanol but not that by DL-homocysteate (DLH) during ejection of naloxone.
currents of naloxone (10-40 nA) which had no effect on, or in two cases enhanced, the action of an amino acid excitant (Fig. 12B).

When ejected for prolonged periods, levorphanol produced a long lasting reduction in acetylcholine sensitivity. This was first observed with cells not directly excited by levorphanol and on which possible effects of the alkaloid on excitatory responses to acetylcholine and an amino acid were being examined. Under these conditions, levorphanol reduced excitation by both acetylcholine and an amino acid and whereas the sensitivity to the amino acid recovered within one minute, that to acetylcholine in 9 cases took 4-11 minutes. This effect is illustrated in Figure 13A. This prolonged recovery time is demonstrated more directly in Figure 13B where cell firing in response to continuous ejection of acetylcholine is shown. With all neurones studied, there was a further decrease in the response to acetylcholine immediately following the cessation of levorphanol ejection, indicating that levorphanol was having a mixed excitant/depressant action at the acetylcholine receptor.

The action of dextrorphan (10-50 nA) was tested on 16 Renshaw cells. The firing, both spontaneous and chemically induced, of 8 neurones was depressed, that of 6 unaffected and with two, weak excitation was observed with the first
Fig. 13: Prolonged reduction in acetylcholine sensitivity of Renshaw cells following ejection of levorphanol.

A. Effect of levorphanol, ejected with currents of 20 nA for 2½ mins, 30 nA for 3 min and 40 nA for 1 min on excitation of a Renshaw cell by alternate ejection of acetylcholine (ACH) and DL-homocysteate (DLH). The time between the second and third sections of this record was 9 minutes.

B. Effect of levorphanol on the firing of a Renshaw cell by continuously ejected acetylcholine (10 nA).
Fig. 14: Depression of cell firing by dextrorphan. Depression during dextrorphan ejection of the excitation of a Renshaw cell by alternate ejection of acetylcholine (ACH) and DL-homocysteate (DLH).
ejection of this compound but not with subsequent
tests. Excitation by acetylcholine and amino
acids were equally reduced by dextrorphan and,
in contrast to results with levorphanol, both
recovered at a similar rate. This effect is
illustrated in Figure 14. The depressant
effect of dextrorphan on Renshaw cells was
often of slower onset and offset than that of
the inhibitory amino acids GABA and glycine.

Levorphanol and dextrorphan were also
tested for effects on the firing of Renshaw
cells in response to a submaximal ventral root
stimulus. Both substances increased the latency
of the individual action potentials, and post-
stimulus histograms prepared from one experiment
are illustrated in Figure 15. Also depicted
are the differing effects of these substances
on the total number of evoked action potentials,
levorphanol increasing and dextrorphan having
little effect on this parameter. Because the
active and inactive isomers had a similar effect
on this response, the antagonist levallorphan
was not tested in this way.

Levallorphan was tested on 29 Renshaw cells
with variable results. With 13, excitation by
acetylcholine and an amino acid were equally
reduced, but a preferential reduction of
excitation by an amino acid occurred with 5
neurones and that by acetylcholine with 4.
Regardless of the type of result, prolonged
Fig. 15: Effects of electrophoretically administered levorphanol and dextrorphan on the responses of a Renshaw cell to a submaximal ventral root stimulus. Each histogram represents the sum of 128 responses, analysed in 200 μs intervals. Ordinates: time in ms after the ventral root stimulus. A. Control. B. 1½ minutes after the start of levorphanol (30 nA) ejection. C. 2 minutes after the cessation of levorphanol ejection. D. 2 minutes after the start of dextrorphan (50 nA) ejection. E. 2 minutes after the cessation of levorphanol ejection.
depression akin to that produced by levorphanol was not observed.

Excitation by morphine was also reduced by levallorphan on all 6 neurones tested, but not specifically as in 5 instances excitation by another substance (L-aspartate with 3 cells, acetylcholine with 2 cells) was also reduced. Reduction of excitation of a Renshaw cell by L-aspartate and morphine following levallorphan ejection is shown in Figure 16A.

The possible interaction between levorphanol and levallorphan was studied on 3 occasions. Currents of levallorphan having minimal effects on excitatory responses to L-aspartate and acetylcholine failed to antagonize their subsequent reduction by prolonged ejection of levorphanol on two cells. With the third, excitation by morphine and levorphanol but not that by DLH, was reversed by levallorphan (Fig. 16B).

2. Intravenous Administration

Six experiments were performed on the effects of intravenous levorphanol on the firing of Renshaw cells following a submaximal ventral root stimulus. Levorphanol was given in a series of divided doses, the total dose varying from 0.5 mg kg⁻¹ to 4.8 mg kg⁻¹. These doses of levorphanol produced an increase in arterial pulse pressure and respiratory depression, but there was considerable variation in the magnitude of these effects between animals.
Fig. 16: Effects of levallorphan on excitation of Renshaw cells by morphine, levorphanol, DL-homocysteate and L-aspartate. A. Reduction of excitation by morphine and L-aspartate (L-ASP) during ejection of levallorphan (10 nA), for 6½ minutes. B. Reduction of the excitation by morphine and levorphanol but not that by DL-homocysteate (DLH) during ejection of levallorphan. Levallorphan was ejected with currents of 10 nA for 3½ minutes, 20 nA for 3 minutes and 30 nA for 2 minutes prior to the middle records being obtained. Ejection (30 nA) was continued for 1 minute beyond these records. Three minutes later the right hand responses were recorded.
The evoked firing of 5 cells was unchanged by intravenous levorphanol, and the responses of the remaining cell slightly enhanced by a dose of 1 mg kg\(^{-1}\) but markedly depressed by a total dose of 4 mg kg\(^{-1}\).

Levallorphan (0.3 mg kg\(^{-1}\)) was given to one animal in which the responses of a Renshaw cell were unaffected by levorphanol and increased the synaptic firing above that of control observations. Levallorphan (0.2 mg kg\(^{-1}\)), administered intravenously to one animal in which the synaptic responses of a Renshaw cell were enhanced by morphine (2 mg kg\(^{-1}\) I.V.), reduced responses to below those of controls.

Naloxone (0.1 mg kg\(^{-1}\)) was given to two animals following levorphanol. In one, both levorphanol and naloxone were without effect on the firing of a Renshaw cell, but in the other the depression of ventral root evoked firing of a Renshaw cell by levorphanol (4 mg kg\(^{-1}\)) was antagonized by naloxone.

Hence whilst the effects of narcotic agonists on Renshaw cell firing was variable, the antagonists reversed an effect regardless of its type.

(f) Physostigmine

Investigations of the interaction of this substance with the action of morphine on Renshaw cells were prompted by the following findings: -

1. The present results suggested that morphine
Fig. 17: The effects of physostigmine on the responses of a Renshaw cell to excitants. The ejection of physostigmine (10 nA) enhances the responses to acetylcholine (ACH) and morphine but not that to DL-homocysteate (DLH).
exerted an excitatory effect by interaction with postsynaptic receptors for acetylcholine. These findings however did not exclude an acetylcholine-releasing action by morphine as proposed by Lodge et al. (1974), and investigations utilizing an anticholinesterase may be a way of distinguishing between these two mechanisms.

2. Physostigmine exhibits analgesic properties in tests such as the inhibition of rat tail flick (Bhargava & Way, 1972).

3. Sub-analgesic doses of physostigmine have been shown to cause previously inactive narcotic antagonists such as nalorphine, but not naloxone, to become agonists in rat tail flick tests (Harris, Dewey, Howes, Kennedy & Pars, 1969).

4. Physostigmine is one of the few non-opiate substances which competes with dihydromorphine for stereospecific central opiate binding sites (Klee & Streaty, 1974).

The effects of ejection of physostigmine on the responses of Renshaw cells to opiate agonists was tested on 9 occasions. For retaining purposes the physostigmine was made up in a NaCl solution and currents employed were low (1-19 nA) to minimize the postsynaptic excitatory actions reported to occur with this substance (Curtis & Ryall, 1966). The excitatory responses to acetylcholine were potentiated by physostigmine on all 9 cells.
Fig. 18: The effects of physostigmine on the responses of a Renshaw cell to opiate antagonists. A. Enhancement of the excitatory responses to acetylcholine (ACH) and nalorphine but not that to DL-homocysteate (DLH) during ejection of physostigmine (3 nA). B. Enhancement of the responses to acetylcholine (ACH) and naloxone (previously negligible effect) but not that of DL-homocysteate (DLH) during ejection of physostigmine (3 nA).
tested and that of an opiate agonist on 8 cells (morphine 4 of 5 cells, levorphanol 4 cells). These enhanced responses occurred without affecting excitant amino acid responses on all occasions and, as shown in Figure 17, were readily reversible.

As previously reported, both the opiate antagonists naloxone and nalorphine are weak excitants of Renshaw cells although high firing rates are not attained by large ejecting currents. Physostigmine (1-5 nA) potentiated the weak excitatory actions both of naloxone (8 of 11 cells) and nalorphine (10 of 18 cells) in the absence of any effects on excitation by DLH, but in all cases that by acetylcholine was increased (Fig. 18).

In all of these tests with physostigmine on Renshaw cells, potentiation, both of acetylcholine and of the opiate agonists and antagonists, was observed as an increased firing rate during their time of ejection. No prolonged firing following the termination of the ejecting current was seen with the recording time constants used.

(g) Oxymorphone

This opiate agonist was tested for its ability to excite Renshaw cells. Since naloxone is the n-allyl derivative of oxymorphone (see Fig. 3), oxymorphone effects were expected to be more specifically antagonized with naloxone.

The actions of oxymorphone (20-60 nA) were tested
initially by observing its effects on the excitatory responses of 19 Renshaw cells to acetylcholine and an amino acid. On 10 occasions the responses to both excitants were enhanced, on seven those to the amino acid alone were enhanced and no effect on either was observed twice. Naloxone was also tested on both types of excitatory response on 10 of these 19 neurones and acetylcholine responses were specifically antagonized on 8 occasions.

It was common for oxymorphone to produce bursts of action potentials during both spontaneous and drug induced periods of cell firing (11 of 19 cells), particularly after prolonged periods or relatively high currents of administration. A weak excitatory action of oxymorphone also became apparent under such conditions. This effect resembled that already described for naloxone, the administration of which was additive to the slowly rising cell firing rate and not antagonistic. Oxymorphone directly excited two Renshaw cells but this effect was not antagonized by naloxone. The number of ventral root evoked action potentials was also increased by oxymorphone (20 nA) on two cells tested but subsequent naloxone (20 nA) failed to antagonize this effect.

(ii) STUDIES WITH NON-CHOLINOCEPTIVE NEURONES

I - UNIDENTIFIED OR CLASSIFIED BY PERIPHERAL FIBRE ACTIVATION

To investigate whether the effects of morphine and the other opiate compounds described on Renshaw cells are peculiar to sites of cholinergic transmission, these compounds were tested at spinal sites where transmission is
### TABLE III - RESPONSES OF DORSAL HORN INTERNEURONES TO MORPHINE AND NALOXONE

<table>
<thead>
<tr>
<th></th>
<th>Sural fibres stimulated to activate each cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A^\alpha$</td>
</tr>
<tr>
<td><strong>Morphine Response</strong></td>
<td></td>
</tr>
<tr>
<td>No effect</td>
<td>6</td>
</tr>
<tr>
<td>Depression</td>
<td>10</td>
</tr>
<tr>
<td>Excitation</td>
<td>2</td>
</tr>
<tr>
<td><strong>Naloxone Response</strong></td>
<td></td>
</tr>
<tr>
<td>No effect</td>
<td>8</td>
</tr>
<tr>
<td>Depression</td>
<td>4</td>
</tr>
<tr>
<td>Excitation</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig. 19: Failure of naloxone to antagonize the depression of firing of a non-cholinoceptive spinal interneurone by morphine and by glycine.
believed to be other than cholinergic.

(a) Morphine and Naloxone Administered Electrophoretically

Morphine and naloxone were ejected electrophoretically in the vicinity of 55 dorsal horn interneurones from the same pipettes used in the studies on Renshaw cells. Of 19 neurones tested with acetylcholine, 5 were weakly depressed and the remainder unaffected. The responses of these neurones to morphine and naloxone did not differ from the cells on which acetylcholine was not tested. Extensive studies on these neurones have shown them not to be excited by acetylcholine (Curtis, Ryall and Watkins, 1966). With 23 of these neurones, the cutaneous fibre types activated by the minimum stimulus adequate for neurone activation was determined. Table III lists the responses of these cells to electrophoretically administered morphine (5-100 nA) and naloxone (5-80 nA).

Neuronal firing, either spontaneous or chemically induced was frequently depressed by morphine and naloxone although this depression was weak in comparison with that produced by glycine and GABA (see Fig. 21).

The ejecting currents of morphine and naloxone to produce equal depressant effects on cell firing did not significantly differ. Nalorphine also depressed the firing of 1 of 2 cells tested.

Naloxone did not antagonize the depression of neuronal firing by morphine on any of the 12 cells tested. Similarly, the inhibitory actions of the amino acids GABA and glycine were unaltered by naloxone on 15 occasions and these effects are illustrated in Figure 19.
Fig. 20: The effects of morphine on blood pressure and the spontaneous and synaptic firing of a non-cholinoceptive spinal interneurone and the reversal of these effects by naloxone. Three intravenous injections of morphine and one of naloxone were given in doses and at times marked by the arrows. A. Spontaneous cell firing obtained prior to injection of morphine and approximately 1 minute after the first and third injections of morphine and the injection of naloxone. B. Post stimulus histograms computed at corresponding times. Cell firing following a stimulus to the ipsilateral sural nerve 2.0 ms before the commencement of each illustrated record was analysed in 200 μs intervals; 128 such responses were summed to produce each histogram. C. Blood pressure measured in a carotid artery at the times of injection of drugs.
The depressant action of glycine is blocked by morphine on both Renshaw cells and interneurones (Curtis & Duggan, 1969), but as with Renshaw cell studies, this effect of morphine was not antagonized by naloxone on six interneurones.

The possible effects of electrophoretically administered morphine on synaptic activation of five spinal interneurones were studied by compiling peri-stimulus time histograms in response to stimulation of a branch of the ipsilateral sciatic nerve. No effect was observed on three of these neurones, the responses of one were depressed and morphine (50 nA) enhanced the synaptic response of the remaining neurone which was also observed to be excited by acetylcholine.

(b) Intravenous Morphine and Naloxone

In three experiments the effects of intravenous morphine were studied on the synaptic activation of interneurones. Morphine in total doses of 7 mg kg⁻¹ reduced the responses of a dorsal horn interneurone to stimulation of a branch of the ipsilateral sciatic nerve in two experiments and had no effect on the third. Naloxone, 0.1 mg kg⁻¹ reversed this depression of evoked firing by morphine and increased spontaneous firing well above that of the control observations in all three experiments. Results from one experiment are shown in Figure 20. Whereas the spontaneous firing (Fig. 20A) of this neurone was progressively depressed by increasing doses of morphine, firing in response to stimulation of the
Fig. 21: Depression of the spontaneous firing of a non-cholinoceptive interneurone by ejection of levorphanol (LOR), dextrophan (DEXT) and glycine. Lack of effect by morphine (MORPH) is also shown.
ipsilateral sural nerve (Fig. 20B) was enhanced by morphine 1 mg kg\(^{-1}\) but depressed by higher doses. Also illustrated is that after naloxone (0.1 mg kg\(^{-1}\)) the evoked firing returned approximately to control levels, but spontaneous firing rose to three times that present before injection of morphine.

In one experiment naloxone was injected intravenously in increasing doses from 0.1-0.4 mg kg\(^{-1}\). No effect was seen on the synaptic or spontaneous firing of the interneurone studied. A subsequent dose of morphine (4 mg kg\(^{-1}\)) also failed to affect cell firing.

(c) Morphinans Administered Electrophoretically

Levorphanol (10-80 nA) did not excite dorsal horn neurones identified by stimulation of A\(\alpha\) fibres of branches of the sciatic nerve and their insensitivity to acetylcholine. Of 12 cells tested, levorphanol weakly depressed the spontaneous or chemically induced firing of 6. Depression by levorphanol (Fig. 21) was of moderately rapid onset and offset and often difficult to distinguish from that due to ejection of sodium ions.

Dextrophan (10-40 nA) was a more consistent and potent depressant of these cells than either morphine or levorphanol (Fig. 21), the firing of all 16 cells tested being depressed. Such depression was of slower onset and offset than that by GABA and glycine (Fig. 21).

(d) Physostigmine

Physostigmine was also tested on cells activated
<table>
<thead>
<tr>
<th></th>
<th>Cholinoceptive Neurones</th>
<th>Non-Cholinoceptive Neurones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphine</strong></td>
<td>- Excites</td>
<td>- Weak Depression or No Effect</td>
</tr>
<tr>
<td></td>
<td>- Increases No. and Latency of Action</td>
<td>- Blocks Glycine</td>
</tr>
<tr>
<td></td>
<td>- Potentials of V.R.R.*</td>
<td>- No Effect on D.R.R.**</td>
</tr>
<tr>
<td></td>
<td>- Blocks Glycine</td>
<td>- I.V. - Depressed D.R.R.</td>
</tr>
<tr>
<td></td>
<td>- Enhances Other Excitants</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Antagonized By Naloxone and DHAE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I.V. Enhances V.R.R. or No Effect</td>
<td></td>
</tr>
<tr>
<td><strong>Naloxone</strong></td>
<td>- No Effect or Very Weak Excitation</td>
<td>- No Effect or Weak Depression</td>
</tr>
<tr>
<td></td>
<td>- No Antagonism of Morphine on V.R.R. or on Glycine Depression</td>
<td>- No Antagonism of Morphine on V.R.R. or Glycine Depression</td>
</tr>
<tr>
<td></td>
<td>- Antagonizes Morphine And Acetylcholine Excitation</td>
<td>- No Antagonism of Morphine Depression</td>
</tr>
<tr>
<td></td>
<td>- I.V. Reverses I.V. Morphine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- I.V. No Effect on V.R.R.</td>
<td></td>
</tr>
</tbody>
</table>

**Morphinans**

- **Levörphanol**
  - Excites
  - Antagonized by Naloxone
  - Long-Lasting Reduction in Acetylcholine Sensitivity
- **Levallorphan**
  - Antagonized Morphine + Acetylcholine but Non-Specifically
- **Dextrorphan**
  - Depressed or No Effect

**Physostigmine**
- Potentiated Morphine and Acetylcholine - Not Amino Acid

**Oxymorphone**
- Weak Excitation Associated with Bursts
- Enhanced Excitants Non Specifically

* Ventral Root Response

** Dorsal Root Response

***Summary only of results up to page 44.
by low threshold dorsal root afferents in an attempt to distinguish differences in its action relative to effects at Renshaw cells. On seven occasions, a current of 2-10 nA had no effect on the depressant activity produced by morphine, naloxone or acetylcholine. As reported previously the depression by morphine was not antagonized by naloxone and this result was not altered by ejection of physostigmine on the one occasion tested. At the currents used, physostigmine was observed to have little effect per se on cell firing rate.

(e) Oxymorphone

Oxymorphone (29-80 nA) was administered in the vicinity of 14 dorsal horn interneurones, having no effect on 9, and weakly depressing the spontaneous firing of the remaining 5 neurones. This effect was not antagonized by naloxone on all occasions tested.

The results reported up to this point have been summarized in Table IV.

(iii) STUDIES WITH NON-CHOLINOCEPTIVE NEURONES

II - CLASSIFIED BY RESPONSE TO NOXIOUS AND NON-NOXIOUS ACTIVATION

It has been reported that morphine, administered electrophoretically in the vicinity of dorsal horn interneurones of cats anaesthetized with α-chloralose, depressed the firing of neurones activated by noxious stimuli but was without effect on cells unresponsive to this type of stimulus (Calvillo, Henry & Neumann, 1974) and that naloxone, similarly administered, antagonized this effect on 2 of 7 neurones. As reported in the preceding section
<table>
<thead>
<tr>
<th></th>
<th>Excited by noxious heat only</th>
<th>Excited by non-noxious mechanical only</th>
<th>Excited by noxious and non-noxious heat</th>
<th>Inhibited by noxious heat</th>
<th>Not activated</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Rexed</td>
<td>IV</td>
<td>1</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Lamina</td>
<td>V</td>
<td>12</td>
<td></td>
<td>1</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>VI &amp; VII</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>TOTALS</td>
<td>1</td>
<td>3</td>
<td>32</td>
<td>5</td>
<td>4</td>
<td>45</td>
</tr>
</tbody>
</table>
of this thesis, morphine administered electrophoretically depressed the firing of about one half of the dorsal horn neurones sampled, but in no case did similarly administered naloxone reverse this effect. As these neurones were not identified by responses to natural stimulation, a further series of investigations was carried out in decerebrate or \( \alpha \)-chloralose anaesthetized animals. Cell positions marked with dye enabled dorsal horn neurones to be classified by laminar location (Rexed, 1954) in addition to responses to noxious and non-noxious stimulation which were produced by the methods already described.

(a) **Distribution of Neurones and Responses to Natural Stimulation**

The responses of neurones in the various layers of Rexed (Rexed, 1954) to noxious and non-noxious stimulation are summarized in Table V. This Table does not give a complete description of cell responses. Cells which were excited by a stimulus in one area but inhibited by a similar stimulus to another area of skin have been classified by their excitatory response. Cells in which an early excitation was followed by inhibition have also been classified by their excitatory response. Receptive field sizes differed considerably. Because of the complexity of responses the terms 'nociceptive' and 'non-nociceptive' have not been used. The Table does emphasize the rarity with which a cell responding to only one sensory modality was encountered.

(b) **Responses to Electrophoretically Administered Morphine**

1. Spontaneous Firing
<table>
<thead>
<tr>
<th></th>
<th>Excited by noxious heat only</th>
<th>Excited by non-noxious mechanical only</th>
<th>Excited by noxious and non-noxious heat</th>
<th>Inhibited by noxious heat</th>
<th>Not activated</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect</td>
<td>1</td>
<td>1</td>
<td>23</td>
<td>2</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Excitation</td>
<td>1</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Depression</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>TOTALS</td>
<td>1</td>
<td>3</td>
<td>32</td>
<td>5</td>
<td>4</td>
<td>45</td>
</tr>
</tbody>
</table>
Table VI summarizes the effects of morphine on neurones classified by response to natural stimulation. It can be seen that morphine was relatively ineffectual as a depressant of cell firing, excitation being seen more commonly, and that there was no correlation between response to skin stimulation and the effects of morphine. Results from one neurone are shown in Figure 22.

Since intravenous morphine has been reported to depress selectively the spontaneous activity of neurones of Laminae I and V and to have little effect on that of neurones of other laminae (Kitahata et al., 1974), Table VII summarizes the responses to electrophoretically administered morphine of cells in layers I to VII. Depression was observed only with cells of the deeper layers.

During these tests, the ejecting current of morphine was increased in a stepwise manner till abnormalities in action potential amplitude and configuration appeared. This occurred with currents of the order of 60 to 80 nA. Excitation by morphine took the form of firing in grouped action potentials (bursts) and was much weaker than that previously observed with Renshaw cells.

2. Excitation by L-Glutamate.

The effects of morphine on excitation by L-glutamate were variable. Of 13 neurones excited both by noxious and non-noxious stimulation of the skin, morphine (30-60 nA) enhanced the responses to glutamate on 5 and
<table>
<thead>
<tr>
<th>TABLE VII</th>
<th>EFFECT OF MORPHINE ON SPONTANEOUS FIRING OF DORSAL HORN NEURONES CLASSIFIED BY LAMINAE OF REXED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>No effect</td>
<td>5</td>
</tr>
<tr>
<td>Excitation</td>
<td>2</td>
</tr>
<tr>
<td>Depression</td>
<td></td>
</tr>
<tr>
<td>TOTALS</td>
<td>5</td>
</tr>
</tbody>
</table>
this was accompanied by an elevation in spontaneous firing and a further increase in excitation by L-glutamate immediately after morphine ejection. Depression of glutamate excitation occurred with 7 neurones but this was associated with abnormalities in action potential amplitude and configuration.

3. Excitation by "Natural" Skin Stimulation

Morphine, ejected with currents of 25-70 nA, reduced firing produced by noxious heat to the appropriate part of the hind limb on only 2 of 37 cells tested. With 9 cells the response was enhanced together with an increase in spontaneous firing. To test for possible specificity in the effects of morphine, 6 cells were activated alternately by noxious heat and by a moving air jet directed at hairs adjacent to a digital pad. In no case was the response to either modality significantly reduced during morphine ejection, this result being illustrated in Figure 22A. This figure illustrates the results from 1 of 3 neurones, the responses of which to both noxious and non-noxious activation were enhanced by morphine at doses great enough to produce irregular firing in bursts.

As a further safeguard that these essentially negative results were not due to a failure to eject an adequate amount of morphine, on five cells depression of firing by glycine was alternated with excitation by noxious heat, as morphine is
Fig. 22: The effect of morphine when administered near the soma of a dorsal horn neurone. A. The responses to noxious heat applied to the fourth digital pad of the left hind limb, are enhanced to a greater extent than those to deflection of adjacent hairs by the ejection of morphine for a total of 7 minutes. That these effects were readily reversible is also illustrated. B. The effects of ejection of morphine are sufficient to block the depression caused by glycine but have negligible effect on the response to noxious heat. Morphine was ejected for a total of 5½ minutes.
known to block the action of glycine (Curtis & Duggan, 1969). In all cases currents of morphine more than twice those adequate to block the action of glycine had no significant effect on activation by noxious heat (Fig. 22B).

(c) Effects of Electrophoretically Administered Naloxone

Ejected with currents of 20-60 nA, naloxone had no effect on the spontaneous firing of 10 cells and weakly depressed that of one. All these cells were activated both by noxious and non-noxious stimuli.

Naloxone reduced excitation by L-glutamate on 4 of 7 neurones, but, following the period of ejection, responses to L-glutamate were enhanced by up to 100% of control values for periods of up to 10 minutes. This effect of naloxone could be a cause of apparent antagonism of the effect of morphine when the latter also reduced excitation by L-glutamate. Thus in tests in which the responses to L-glutamate had been depressed by morphine, the simultaneous ejection of naloxone either had no effect or produced further depression, but the removal of naloxone, whilst morphine was still being ejected, resulted in responses greater than those prior to naloxone ejection.

Naloxone, ejected alone, did not modify the firing of cells by noxious heat.

Electrophoretically administered naloxone did not antagonize the effect of similarly applied morphine in any of the tests, namely depression of excitation of L-glutamate (3 cells), enhanced
excitation by L-glutamate (3 cells), reduced excitation by noxious heat (1 cell), enhanced excitation by noxious heat (3 cells).
DISCUSSION

The results of the initial two studies indicate that electrophoretically administered morphine excited neurones by activating nicotinic receptors for acetylcholine. The evidence supporting this conclusion was provided by the following:

1. Distribution of nicotinic receptors. Nicotinic receptors are present on Renshaw cells (Curtis & Eccles, 1958; Curtis & Ryall, 1966) but not on other spinal interneurones (Curtis, Ryall & Watkins, 1966). Investigations carried out in this laboratory have demonstrated that ventrobasal thalamic neurones, which also possess nicotinic receptors (Andersen & Curtis, 1964) are excited by morphine, whereas neurones of the cerebral cortex possessing receptors with muscarinic properties (Krnjević & Phillis, 1963a; Crawford & Curtis, 1966) were not excited by morphine. The distribution of nicotinic receptors therefore parallels that of excitation by morphine and this series of results have been published collectively (Duggan, Davies & Hall, 1976).

2. Naloxone antagonized excitation by acetylcholine and nicotine but not that by acetyl-β-methylcholine.

3. Excitation of Renshaw cells by morphine was readily antagonized by DHβE.

The excitatory action of morphine at this site could result from any of the following possible mechanisms:

1. An enhancement of the action of tonically released acetylcholine either through an inhibition of cholinesterase or sensitization of postsynaptic receptors.

2. A release of acetylcholine from presynaptic terminals.
3. Activation of postsynaptic receptors for acetylcholine.

Inhibition of cholinesterase can probably be excluded as a possible explanation by the finding that nalorphine, which did not excite Renshaw cells, is as potent as morphine as an inhibitor of rat brain cholinesterase (Dewey, Harris, Howes, Kennedy, Nuite & Hayhurst, 1969). In addition, no detectable prolongation of the action of acetylcholine or any other excitant was observed during morphine ejection, and the action of an excitant amino acid was usually enhanced to a similar extent as that of acetylcholine. Several studies have indicated a lack of correlation between inhibition of cholinesterase and narcotic analgesic potency (Young, Van der Ploeg, Featherstone & Gross, 1955; Dewey \textit{et al.}, 1969; Szerb, 1957).

A release of acetylcholine from presynaptic terminals by morphine administered electrophoretically has been proposed by Lodge \textit{et al.}(1974) after their observation that morphine enhanced the action of acetylcholine more than that of DLH on Renshaw cells of the rat. Morphine also increased the latency of the ventral root evoked action potentials and these authors proposed that some of these effects of morphine could be explained if it caused a partial depolarization of both the Renshaw cell and the presynaptic terminal. This latter effect, which has been proposed as a mechanism of presynaptic inhibition (Eccles, 1964; Schmidt, 1971), could account for the increased latency of the ventral root response whereas the increase in the number of action potentials could be due to an increase in the postsynaptic membrane excitability, and thus the sensitivity to acetylcholine, by morphine. A presynaptic depolarizing effect would increase spontaneous release of transmitter (Del
Castillo & Katz, 1954; Liley, 1956) and this might possibly account for the elevation in Renshaw cell excitability which was observed. Indeed, such a mechanism of action of morphine cannot be completely ruled out from the findings reported here. To pursue the question of the mechanism of action of morphine, the experiments with physostigmine were performed. These results, which are discussed below, provide no decisive information concerning this problem.

In the present investigations, the reduction of the effects of electrophoretically administered acetylcholine and nicotine by naloxone is almost certainly a postsynaptic effect at receptors for acetylcholine. The simplest explanation for a parallel reduction by opiate and cholinergic antagonists of the excitation by morphine and acetylcholine is that morphine is also acting postsynaptically. If, however, morphine releases presynaptically stored acetylcholine and naloxone antagonizes this action, then excitation by morphine should be more readily reduced by naloxone than excitation by acetylcholine. This was not observed.

The results with levorphanol are consistent with an excitatory effect at nicotinic receptors, an action not observed with dextrorphan. The prolonged decrease in acetylcholine sensitivity also observed with levorphanol, however, is similar to the effect of atropine ejected electrophoretically (Krnjević & Phillis, 1963b). Whilst levallorphan showed naloxone-like activity on a few Renshaw cells, in most cases this narcotic antagonist non-specifically reduced the activity of all excitants tested. In microelectrophoresis studies, such activity is also shown by procaine (Curtis & Phillis, 1960). These results with morphinans, therefore, showed similarities to those obtained with
morphine and naloxone but also significant differences.

Activity shown by morphinans in other experiments may be relevant to these differences. Both levorphanol and levallorphan are toxic to cell cultures and inhibit RNA synthesis (Simon, 1971), and all three of the morphinan compounds tested in the present investigations are more potent than morphine as local anaesthetics at the squid axon (Simon & Rosenberg, 1970). Dingledine & Goldstein (1973) have also shown that prior naloxone administration to mice reduces the lethality of LD₉₈ doses of levorphanol, but that it is ineffective at higher doses of levorphanol and of dextrorphan. In addition, levorphanol is approximately one thousand-fold more lipid-soluble than morphine (Von Cube et al., 1970), a property which may limit its distribution when ejected from a micropipette. This characteristic may result in relatively high concentrations in the vicinity of the tip of the micropipette and could explain both the inability to attain high firing rates with levorphanol and the predominantly local anaesthetic type of activity exhibited by levallorphan.

Morphine also has an excitatory effect on neurones of the rat brainstem (Bradley & Dray, 1974), and whereas no correlation between excitation by morphine and by acetylcholine was found, a subsequent report from that laboratory (Bramwell & Bradley, 1974) suggests that such a correlation exists. Nicotinic receptors for acetylcholine can be readily demonstrated in the brainstem of the decerebrate rat (Duggan, Headley & Lodge, 1975), but not in the cat (Duggan & Game, 1975). Bramwell & Bradley, (1974) reported that naloxone did not block the excitation of brainstem neurones of the rat by morphine but blocked the depression which followed excitation, but the specificity of
this effect in the absence of control responses to other depressants cannot be evaluated. The response to morphine was tested after, but not during, the ejection of naloxone and this may explain the failure to observe antagonism of morphine's excitation, since in the results presented here, naloxone's effects were rapidly reversible.

Other studies of the effects of morphine administered electrophoretically show differences from the present results. A depression by morphine of the activity of spinal interneurones of Laminae IV, V and VII of the cat has been reported (Dostrovsky & Pomeranz, 1973). These authors interpret this effect, which was observed as a reduction of the excitatory responses to L-glutamate, as a blockade of postsynaptic L-glutamate receptors, but since this is not observed with Renshaw cells, it may be that morphine depresses cell firing by another mechanism. Furthermore, the failure of naloxone to antagonize this effect casts doubt on its relevance to the actions of systemic morphine. Intracellular recording from feline spinal neurones has also demonstrated that morphine blocks the depolarization due to L-glutamate (Zieglgänsberger, Satoh & Bayerl, 1975). These authors found that morphine depressed 60% of the cortical neurones studied in the rat and could also antagonize the excitatory effects of L-glutamate and acetylcholine on these neurones. Both of these effects, in the cat and in the rat, could be reversed by naloxone, also administered electrophoretically.

The studies reported here with physostigmine suggest that, if it acted purely as an anticholinesterase, then morphine and levorphanol excite neurones by release of acetylcholine, and that naloxone and nalorphine also have this action. Enhancement of
the action of acetylcholine seen at the same time however, would suggest that this is not so. An interaction between anticholinesterases and nicotinic receptors of Renshaw cells has been proposed (Curtis & Ryall, 1966) to account for the lack of correlation between enhancement and prolongation of the action of acetylcholine, the depression by edrophonium of acetylcholine sensitivity and the block by DHβE of excitation produced by these substances.

The ability of physostigmine to affect the responses of Renshaw cells to opiate compounds but not those of noncholinceptive neurones might be taken to suggest a similarity between the receptor for acetylcholine and the opiate receptor studied in the binding experiments (Terenius, 1973), but other effects not always related to cholinceptive neurones have been reported for this substance. Physostigmine has been reported to have predominantly excitatory effects on a proportion of brainstem neurones of the cat which could not be correlated with responses to acetylcholine (Salmoiraghi & Steiner, 1963; Bradley, Dhawan & Wolstencroft, 1966). Although relatively high concentrations were required to produce these effects, the responses of brainstem neurones to acetylcholine were shortened in latency and prolonged in offset in the presence of physostigmine.

Spehlmann (1963) has also reported that cortical neurones were excited by electrophoretically administered physostigmine alone as well as this substance enhancing the effect of acetylcholine.

The report by Harris et al., (1969) that nalorphine but not naloxone exerts agonist activity in the presence of low concentrations of physostigmine in tail flick tests does not correlate with the results of the present type of experiment. The weak excitatory effects of both naloxone and nalorphine were
enhanced by physostigmine. It may be that this effect of physostigmine is unrelated to anticholinesterase properties in which case it would be not amenable to testing by the methods employed here.

Physostigmine enhances the inhibition of trigeminal motoneurones by impulses in cutaneous nerves (Iwata, Sakai & Deguchi, 1971) and such an action may be relevant to the activity of this substance in procedures which purport to test for analgesic activity.

The studies with oxymorphone and Renshaw cells indicate that it has several properties similar to morphine, namely the ability to potentiate the actions of other excitants and to be excitant, albeit weakly, itself. A major difference however, was the failure of naloxone to antagonize the effects of oxymorphone as well as the pronounced ability of this substance to produce bursts of action potentials. Hence, while oxymorphone and naloxone did exert different effects at the Renshaw cell receptor, they did not behave as agonist and antagonist compounds, a result which argues against the relevance of these effects to those of systemic opiates.

The relevance of the investigations described here to the central effects of systemically administered opiates is rendered difficult by a lack of knowledge of concentrations obtained by electrophoretic administration. Four effects of morphine were consistently observed when administered electrophoretically on spinal cord neurones -

1. An enhancement of the number of action potentials evoked by a ventral root stimulus, as well as a decrease in the latency of the second and subsequent action potentials.

2. Antagonism of the depression exhibited by glycine.
3. Depression of dorsal horn interneurones.
4. Excitation of Renshaw cells and enhancement of excitants.

Of these effects only the latter, excitation of Renshaw cells as a result of interaction with postsynaptic acetylcholine receptors, could be antagonized by similarly administered naloxone. If activity at nicotinic receptors therefore, is of importance to the central effects of systemically administered opiates, then drugs possessing activity at such receptors could be expected to influence these effects. A recent report by Phan et al. (1973) has demonstrated the antinociceptive properties of nicotine. As presented in the introduction, however, the literature concerning the effects of cholinergic substances on analgesia, tolerance and withdrawal is controversial, as is that concerning morphine and the levels and release of acetylcholine.

The lack of activity of naloxone when administered systemically (Blumberg et al., 1961) would seem to be evidence of a major fault in the proposal that the activity of opiate compounds is a result of interaction with the postsynaptic receptors for acetylcholine. It must be recalled however, that with the exception of Renshaw cells, a role for central nicotinic receptors in synaptic transmission has not been convincingly demonstrated (but see McCance, Phillis & Westerman, 1968). Goldstein (1974) has proposed that if narcotic agonists and antagonists occupy the same receptor site, then the lack of central effects by naloxone implies that morphine cannot act by interfering with the action of a normally active transmitter. If such receptors are located at sites other than functional synapses, then the acetylcholine-antagonist property of naloxone
demonstrated here is consistent with the relative lack of central effects when this substance is given alone.

Consistent with Goldstein's hypothesis is the recent demonstration of a series of pentapeptides ("endorphins") extractable from brain tissue (Hughes, 1975; Terenius & Wahlström, 1975), possessing analgesic properties (Büscher et al., 1976) and able to displace opiates in binding experiments (Hughes et al., 1975). These peptides, the active parts of which have been suggested to correspond with active groups on the morphine molecule (Büscher et al., 1976), may be released under particular conditions, e.g. stress to the organism, and if so, activity at receptors identical to those where opiates interact but distinct from those at normal functional synapses, could be reasonably invoked.

The results presented in the later study do not confirm the finding of Calvillo, Henry & Neumann (1974) that morphine administered electrophoretically to dorsal horn neurones depresses only those activated by painful stimulation. These authors reported a higher proportion of neurones not activated by painful stimulation, about 50% of their total number of neurones, than the present and other studies (Handwerker, Iggo & Zimmerman, 1975; Price & Browe, 1973, 1975). Such a difference however, cannot explain the difference in results.

The findings presented in the second study of this section reported a high proportion of the neurones studied to be depressed by morphine and this was probably due to the sampling of neurones of deeper layers of the dorsal horn. Deeper situated neurones of the present studies were more commonly depressed by morphine (Table VII).

The essentially negative results of the latter investigations
cannot be explained by failure to eject an adequate amount of morphine. Currents used were in excess of those necessary to block the action of glycine (Curtis & Duggan, 1969; Dostrovsky & Pomeranz, 1973) and were increased until abnormalities in action potential amplitude and configuration appeared. An elemental analysis (performed by Microanalytical Service of this University), and the effects on blood pressure after intravenous administration and their reversal by naloxone, indicated the adequacy of the purity and potency of the sample of morphine used.

Kitahata et al. (1974) have reported that intravenous morphine 0.5-2.0 mg kg\(^{-1}\) depressed the spontaneous activity of neurones of Rexed Laminae I and V and not those of neurones in Laminae IV and VI. It has been reported (Christenson & Perl, 1970; Hillman & Wall, 1969) that neurones in Laminae I and V respond principally to noxious stimuli while those in Laminae IV and VI are responsive to non-noxious stimuli. Results from the present and other studies (Handwerker et al., 1975; Price & Browe, 1975) suggest that this is an oversimplified concept as in the present experiments there was no correlation between the effects of morphine and laminar location except perhaps for the deep Laminae VI and VII. It becomes obvious that care must be taken when interpreting the effects of an intravenously administered drug at the single cell level.

While the present results do not lend support to the body of evidence implicating a spinal site of action, it must be borne in mind that the electrophoretic technique, which administers drugs to the extracellular environment of the neuronal soma, may be unable to duplicate the conditions consequent upon intravenous administration. Hence the inability to demonstrate depression by electrophoretically administered morphine of nociceptive
responses of dorsal horn neurones cannot definitely provide support for or against a spinal site of action for morphine.
Introduction

The findings of the previous section indicated that morphine, administered near cell bodies of dorsal horn neurones, did not produce effects which could be related to those of systemic morphine on responses to noxious or non-noxious stimulation. This result has two explanations - either the morphine-like compounds do not act in the dorsal horn or they were not administered to the appropriate site.

Since in the spinal cat, intravenous morphine reduces the activation of dorsal horn neurones by nociceptive afferents (Le Bars, Menetrey, Conseiller & Besson, 1975), the latter explanation, failure to administer the opiate to the appropriate site, was considered the more likely.

That receptors on dendrites might be relevant to the action of putative transmitter substances and possibly of drugs has been suggested by several studies (Diamond, 1968; Dudar, 1974; Ransom & Nelson, 1975; Yamamoto, 1976). It seemed possible then, that receptors distant from the cell body and related to the interactions of opiates might conceivably be present on Laminae IV and V neurones. It is these neurones which receive a large proportion of cutaneous and nociceptive afferents (Schimert, 1939; Wagman & Price, 1969; Handwerker, Iggo & Zimmerman, 1975; Price & Browe, 1975) and their dendritic processes project dorsally to the substantia gelatinosa region of the dorsal horn (Laminae II and III; Cajal, 1909; Szentágothai, 1964).

The substantia gelatinosa is a region cytoarchitecturally distinct from the rest of the dorsal horn and contains an
Fig. 23: A diagramatic representation of the arrangement of the two electrodes in the spinal cord. The vertical electrode which was positioned in the substantia gelatinosa region of the spinal cord was used for administration of compounds. The other electrode penetrated the spinal cord at an angle and located neurones in laminae IV or V which were appropriate for extracellular recordings (see Chapter II). The dendritic relationship between lamina IV and substantia gelatinosa neurones and with primary afferent terminals is also depicted diagramatically.
abundance of axo-axonic synapses (Szentágothai, 1964; Réthelyi & Szentágothai, 1965; Scheibel & Scheibel, 1968; Mannen & Sugiura, 1976). It is this property of the substantia gelatinosa which has led Szentágothai (1964) to suggest that it may be involved in the "modulation of impulse transmission through the larger neurones of the dorsal horn". Melzack & Wall (1965) have proposed that the substantia gelatinosa serves as a "gate control system" which modulates afferent nociceptive information. The proposal by these workers that the axo-axonic synapses of the substantia gelatinosa might be the basis of a mechanism of presynaptic inhibition of nociceptive primary afferents has not been demonstrated definitively from anatomical studies (Réthelyi & Szentágothai, 1965; Szentágothai, 1968; but see Réthelyi & Szentágothai, 1969).

The present investigation was an endeavour to influence the responses of Laminae IV and V neurones to noxious and non-noxious stimuli by the electrophoretic ejection of morphine, naloxone and other compounds in the region of substantia gelatinosa using the "two-electrode" technique outlined in Methods (Chapter 2, see Fig. 23). All animals were anaesthetized with chloralose and were artificially respired.
Fig. 24: Depression by morphine of nociceptive but not mechanoreceptor activation of a dorsal horn neurone and reversal of this effect by intravenous naloxone. The neurone was located in spinal lamina V and was activated by noxious heat to the third digital pad of the left hind limb and deflection of adjacent hairs: separation between tips of drug administering and recording micropipettes 340 μm. The lower traces are pen recordings of cell firing in response to noxious heat and hair deflection and the upper record is a graph of response amplitude with respect to time of each stimulus. Spontaneous firing is also plotted. The bars above the graph indicate the times of ejection of morphine. The letters A, B, C and D indicate the times at which the lower tracings were recorded.
RESULTS

(a) **Effects of Morphine on the Responses of Lamina IV and V Neurones to Noxious and Non-noxious Stimulation**

Morphine, ejected in the region of the substantia gelatinosa with currents of 30 to 250 nA, reduced the responses to noxious stimulation of 12 of 16 Laminae IV or V neurones excited by alternate noxious and non-noxious stimuli. With one neurone, both responses were depressed and with the remaining 3 neither was affected. Of the 12 neurones, the activation of which by the nociceptive stimulus was reduced by morphine, the response to non-nociceptive stimulation was not significantly changed in 7 and increased above controls in 5. With a further 4 neurones, nociceptive responses alone were studied and morphine reduced this activation of 3. In Figure 24, for the period between A and C, the ability of morphine to reduce the response of one neurone to nociceptive stimulation, independent of effects on spontaneous firing or that in response to non-nociceptive stimulation, is illustrated by a plot of response amplitude with respect to time. The actual responses A and B in the lower part of this figure, illustrate this action more directly (see legend for details).

The temporal relationships of the effects of morphine were different from those usually observed when compounds are administered electrophoretically in the vicinity of cell bodies. Firstly, there was considerable variation in the time to onset of the reduction of nociceptive activation, this ranging from 4-35 minutes. Since the relative positions of recording and drug administering pipettes varied from neurone to neurone, then the administration of varying doses of morphine would be expected...
Fig. 25: Effect of distance between drug administering and recording pipettes on the dose of morphine, ejected in the substantia gelatinosa region, necessary to reduce nociceptive activation of dorsal horn neurones. Ordinate: charge in µ coulombs passing through the morphine containing pipette up to the time of reduction of nociceptive firing. Abscissa: distance in µm between the tips of the drug administering and recording micropipettes.
to take different lengths of time to achieve the appropriate concentration conditions at the structures within the substantia gelatinosa region which were being affected. The analysis shown graphically in Figure 25 indicates that this time to effect was not related simply to the time for morphine to diffuse to and have effects at the cell body - a possible site of action ruled out by the previous study. In order to construct this graph the dose of morphine administered was calculated by multiplying the current passed through the morphine containing barrel by the time for reduction in the response to nociceptive activation. This calculation makes the assumption that the transport number for morphine did not greatly differ between pipettes. This dose was then plotted against the distance separating the tips of the recording and drug administering micropipettes and it can be seen that these variables are not closely correlated. Neurones to which naloxone had been administered some time previously to morphine were omitted from this analysis.

Secondly, the reduction of nociceptive activation by morphine was long lasting but, because its abrupt reversal by intravenous naloxone was considered necessary evidence of specificity, the total duration of this depression was not determined with any accuracy. In five experiments this depression was still unchanged at 17, 50, 12, 56 and 21 minutes after cessation of morphine ejection in the SG region. That morphine was still active at this time was shown by the rapid reversal of this depression by intravenous naloxone in Figure 24 and also in Figure 26.
**Fig. 26:** Prolonged effect of morphine, ejected in the substantia gelatinosa region in reducing nociceptive activation of a dorsal horn neurone and reversal of this effect by naloxone administered both electrophoretically and intravenously. The neurone was located in spinal lamina IV and activated by noxious heat to the fourth digital pad of left hind limb. Response amplitude and spontaneous firing are plotted with respect to time. The times and currents in nA used to eject morphine and naloxone are indicated by bars. Between 100 and 110 minutes from the start of recording DL-homocysteate was ejected in the vicinity of the cell body, but these responses have been omitted for clarity.
(b) **Effects of Naloxone**

(i) Administered Electrophoretically in Association with Morphine.

Naloxone, administered electrophoretically with similar currents and from the same pipettes used to administer morphine, reversed the depression by morphine of the nociceptive activation on all 12 neurones tested (Fig. 26 and 27). In these tests, naloxone ejection was added to that of morphine after the latter drug had depressed nociceptive activation and morphine ejection continued beyond that of naloxone until this response had once more been depressed (Fig. 26 and 27).

Two types of result were obtained. With 4 neurones, the responses of which to nociceptive stimulation had been depressed by morphine, naloxone restored these responses to control levels without significant effect on non-nociceptive or spontaneous firing. With 8 other neurones, naloxone enhanced firing to above control levels and increased spontaneous firing as well as that produced by non-nociceptive stimulation on 6 occasions when this was concurrently tested. As illustrated in Figure 27, the increased firing rates attained during naloxone ejection were not brought about merely by an elevation in spontaneous firing; the total number of action potentials in response to each period of stimulation was increased, the increase being greater for nociceptive than for non-nociceptive stimuli. Figures 26 and 27 also show that for morphine to overcome the effects of naloxone, large
Fig. 27: Depression by morphine of nociceptor but not mechanoreceptor induced firing of a dorsal horn neurone and reversal of these effects by electrophoretic naloxone. The neurone was located in spinal lamina V and morphine and naloxone were ejected in the substantia gelatinosa region. Separation between drug administering and recording pipettes was 330 μm. The arrangement of plotted cell responses and tracings of cell firing are as in Figure 24. The ability of electrophoretically administered naloxone to restore the response to noxious heat to above control values is also illustrated and this effect was also present but to a lesser degree with responses to hair deflection.
ejecting currents for prolonged periods were required, and Figure 26 also illustrates that the effects of electrophoretically administered naloxone in the substantia gelatinosa region can be duplicated with intravenous administration.

(ii) Administered Intravenously

Administered following the cessation of morphine ejection, intravenous naloxone abruptly reversed, in all 4 experiments, the depression of nociceptive activation by morphine. In three experiments the first dose of naloxone was effective (0.1, 0.3 and 0.3 mg kg\(^{-1}\)) and in the remaining one, two doses of 0.3 mg kg\(^{-1}\) were necessary. The results from two of these experiments have been shown in Figures 24 and 26, and to ensure that changed responses following naloxone in Figure 24 were not merely due to recovery from previous morphine, a second dose of morphine was administered in the region of the SG just prior to the intravenous naloxone. The abrupt nature of the change in the response to nociceptive stimulation in these and other experiments confirms that this effect was indeed produced by the intravenous antagonist.

Naloxone, administered intravenously (0.5-1.2 mg kg\(^{-1}\)) during the continued ejection of morphine, reversed the depression of nociceptive activation by morphine in 3 of 6 experiments. All of these results were obtained from cells on which the effects of morphine had been previously antagonized by electro-
phoretically administered naloxone, and on which prolonged ejection of morphine was necessary to overcome the effect of naloxone. In the tests where intravenous naloxone had no effect, the approximate amounts of morphine previously ejected were 150 nA for 90 min, 200 nA for 105 min and 200 nA for 150 min. For those in which intravenous naloxone reversed the effects of morphine the amounts were 200 nA for 120 min, 30 nA for 70 min and 60 nA for 90 min.

(iii) Effects of Electrophoretically Administered Naloxone Alone

As pointed out in previous discussion, naloxone is considered amongst the "purest" of opiate antagonists in the sense that it has minimal agonist activity in animals (Blumberg, Dayton, George & Rapaport, 1961) and man (Lunn, Foldes, Moore & Brown, 1961; Blumberg & Dayton, 1972). In the present experiments, naloxone administered after morphine often raised the spontaneous, nociceptive and non-nociceptive firing to above control levels. It therefore became necessary to determine the possible excitant properties per se of this compound when administered electrophoretically in the SG region.

Eleven neurones were tested in this way, 8 in lamina IV, 3 in lamina V.

When ejected with currents similar to those required to reverse the effects of morphine, naloxone had no effect on the firing of 8 neurones. With 2,
the levels of each of the three firing parameters were elevated to just above controls and with one, the spontaneous and nociceptive activation of which alone were studied, these two types of firing were also elevated.

(c) Effects of Amino Acids

To test the possibility that morphine might be acting by exerting a predominantly excitant or depressant effect on neurones of the substantia gelatinosa, excitant (L-glutamate, DL-homocysteate) and depressant (glycine, GABA) amino acids were also ejected in this region. A considerable variation in effects of these substances on spontaneous firing and on the responses to nociceptive and non-nociceptive stimulation was observed.

An excitant amino acid was administered to the substantia gelatinosa on 13 of 16 occasions when morphine was effective in reducing nociceptive responses. With 4 neurones no effect on any firing pattern was observed even after ejections of up to 200 nA for 13 minutes; with 3 the spontaneous firing was variably increased but the responses to peripheral stimulation were unaffected; with 4 spontaneous firing and nociceptive but not non-nociceptive responses were increased; with one all three firing patterns were increased and with another both the spontaneous and nociceptive afferents were reduced. The 3 neurones, the responses to nociceptive stimulation of which were unaffected by morphine, were all excited by an excitant amino acid but with no consistent effect on responses to skin stimulation.

The depressant amino acids were almost without effect. Of four neurones on which morphine had reversed responses to
nociceptive stimulation, glycine or GABA had no effect on 3 (200 nA for 5 mins) and depressed all the patterns of firing of the remainder (30-100 nA, 15 mins). Of 11 other neurones on which glycine or GABA were tested, effects were seen on only 2, both with GABA, with one other spontaneous alone was reduced and with the other spontaneous and the nociceptive response were reduced.

On no occasion was specific reduction of nociceptive response observed following the ejection of amino acids.

Effects with amino acids, when observed, also exhibited a time course different to those of morphine. Although the time of onset of any detectable effect was frequently prolonged when compared to administration near the cell body, reversal of these effects was usually evident within 2 minutes.
DISCUSSION

The present investigations have revealed an action of electrophoretically administered morphine in the spinal cord which shows selectivity in the sensory modality affected, is reversible by similarly or intravenously administered naloxone and which is exerted at sites distant from the cell body of the neurones studied. That these observations are relevant to the actions of morphine administered systemically, is supported by the following considerations:

1. Sensory Modality Selectivity

The ability of morphine to alleviate pain in humans has long been known (for quantitative study see Wolff, Hardy & Goodell, 1940). That it does so independent of effects on other sensations, also receives support from the clinical literature (Wikler, Goodell & Wolff, 1945; Eddy, 1941).

Animal studies have shown that intravenous morphine-like compounds have selective actions at the spinal cord in reducing transmission in small diameter afferent fibres. Le Bars et al. (1975), using a combination of nociceptive natural stimulation and electrical stimulation of large diameter cutaneous afferents, found that intravenous morphine (2 mg kg\(^{-1}\)) depressed the responses of spinal lamina V neurones to the noxious stimulus alone. A similar result had been found previously with phenoperidine (Besson et al., 1973) and fentanyl (Iwata & Sakai, 1971). Other studies measuring the effects of intravenous morphine on spinal reflexes also report that it more readily reduces those produced by impulses in small rather than large diameter.

The present experiments, by demonstrating that morphine administered in the region of the substantia gelatinosa was specific in reducing firing by nociceptive but not by mechanoreceptor afferents, thus show good correlation with the effects of systemic morphine. That this apparent selectivity was a result of the failure of morphine to reach the terminals of mechanoreceptor afferents finds no support in the anatomical literature. Although large diameter primary afferents curve over the dorsal horn and enter the substantia gelatinosa from the ventral side (Cajal, 1909), and unmyelinated fibres enter directly from Lissauer's tract (Ranson & Billingsley, 1916; Scheibel & Scheibel, 1968), the terminals of both types are apparently widely distributed throughout the substantia gelatinosa (Szentágothai, 1964).

2. Antagonism by Naloxone

While the demonstration that electrophoretically administered naloxone antagonized the effects on nociceptive responses of similarly administered morphine might suggest relevance to the actions of systemic morphine, the unknown concentrations of substances when ejected by electrophoresis prevent such a demonstration from being completely definitive. The additional demonstration however that intravenous naloxone antagonized the actions of morphine establishes more firmly the relevance of the observations. Indeed, it is important when demonstrating the relevance of these effects to those following systemic administration, to
stress the finding that intravenous naloxone was effective in reversing the effect of morphine with doses as low as 0.1 mg kg$^{-1}$. Doses of this order are effective in reversing the effects of analgesic doses of morphine in the cat (Jurna & Grossman, 1976) and the dog (McLane & Martin, 1967a).

3. Enhancement of Responses by Naloxone to Above Control Levels

The ability of electrophoretically administered naloxone in the present studies to enhance the depressed responses to nociceptive stimulation following morphine to above control levels in two-thirds of the neurones studied is an action which is similar to that reported for intravenous naloxone in the literature, and therefore might emphasize further the relevance of the present results. Naloxone when given intravenously to reverse effects of morphine has been reported to elevate responses above control levels at low doses (Le Bars et al., 1975; Jurna & Grossman, 1976). In the present study such an effect was accompanied by an increase in cell excitability as shown by increased spontaneous firing and an enhanced response to mechanoreceptor stimulation. As naloxone alone did not exhibit such activity it appears that this compound has an action, on neurones exposed to morphine, different to that on neurones not previously exposed. Whether this phenomenon is in any way an analogy at the single cell of precipitated abstinence (Wikler et al., 1953) is a matter for speculation. Although in this context the times of exposure were relatively short, recent reports have shown that in mice naloxone is able to precipitate a jumping withdrawal symptom within 10 minutes of morphine administration.
(Kosersky, Harris & Harris, 1974) and that the number of opiate binding sites is increased five minutes after implantation of a morphine pellet (Pert & Snyder, 1976).

The long time of onset and duration of the effect of morphine in the region of the substantia gelatinosa differs from any other report of an effect of this substance administered electrophoretically to dorsal spinal neurones. The depression of firing reported by Dostrovsky & Pomeranz (1973); Calvillo, Henry & Neumann (1974); Satoh, Zieglgänsberger, Fries & Herz (1974); Duggan, Davies & Hall (1976); and Gent & Wolstencroft (1976) was rapidly reversible. Depression of brainstem neurones (Bramwell & Bradley, 1974) and excitation of Renshaw cells (previously reported here) is also rapidly reversible. The relatively slow onset of effect by morphine may in part be due to the time required to achieve appropriate concentrations of morphine at the relevant sites in the substantia gelatinosa. It may well be that an interaction of morphine at many sites within the substantia gelatinosa is needed before effects can be seen. If the axonal arborization of neurones of the SG and the dendritic processes of lamina IV neurones are as widely ramifying as the literature suggests (Szentágothai, 1964; Mannen & Sugiura, 1976), then this possibility cannot be ruled out.

A very slow rate of removal of morphine is unlikely to explain its prolonged action since the effects, when present, of DL-homocysteic acid similarly administered decayed relatively quickly. When administered near cell bodies the effects both of DL-homocysteate and morphine are rapidly reversible, suggesting that both compounds are
readily removed from the extracellular environment. Other workers have commented on the long duration of effect of morphine given intravenously. Le Bars et al., (1976) reported that intravenous morphine caused a depression of the spontaneous and evoked firing of dorsal horn neurones which lasted greater than 40 minutes, the depression of the flexor reflex of the dog by intravenous morphine which was observed by McClane & Martin (1967b) to become maximal after 1 hour, remained at that level for over 4 hours. Administered intraventricularly to rabbits, the analgesic action of morphine is more prolonged than when administered intravenously, a difference attributed to the difficulty with which the hydrophilic morphine molecule penetrates nervous tissue (Herz & Teschemacher, 1971). This latter property is unlikely to explain the prolonged effect of morphine in the present results since effects observed following the administration of DL-homocysteate, which is readily soluble in water, were rapidly reversible. These considerations suggest a slow dissociation from the opiate receptor in the substantia gelatinosa.

The anatomical localization of the opiate receptor studied in the present experiments is not known. Autoradiographic studies of systemically injected $^3$H-diprenorphine (Pert, Kuhar & Snyder, 1975) have shown the substantia gelatinosa to be amongst the highest opiate-binding sites in the central nervous system of the rat. The method was not adequate to determine the fine structural basis of binding but, in all labelled regions, silver grains were located between cell bodies and not over them. Such a finding correlates well with the previous studies where
administration of morphine to the cell body regions of dorsal horn neurones activated by noxious stimuli gave results bearing no relation to the effects of systemic morphine. Studies on the monkey have shown the upper dorsal horn (which includes the substantia gelatinosa) to be a region of high stereospecific binding of opiates, and that sectioning of the dorsal roots results in a significant reduction in such binding (Lamotte, Pert & Snyder, 1976). This finding is consistent with the localization of receptors on terminals of primary afferent fibres but cannot exclude loss of receptors associated with trans-synaptic degeneration of neurones.

Because of the complexity of organization of the substantia gelatinosa, any of the following mechanisms could explain the effects of morphine observed in the present experiments:

1. Inhibition of transmitter release from the terminals of nociceptive afferents. This could be produced by block of conduction in fine terminals or activity at axo-axonic synapses known to be abundant in the substantia gelatinosa (Rethelyi & Szentágothai, 1969).

2. Block of the postsynaptic action of the transmitter released by nociceptive afferents. Although this transmitter has not been identified, some evidence has accumulated which implicates a transmitter role for substance P an undecapeptide at the terminals of small diameter primary afferents (Hökfelt, Kellerth, Nilsson & Pernow, 1975; Otsuka, Konishi & Takahashi, 1975). The relevant receptors could be located on the dendrites of neurones of deeper spinal laminae or
on neurones of the substantia gelatinosa.

3. Depression of excitatory interneuronemes or excitation of inhibitory neurones of the substantia gelatinosa. The results with excitatory and inhibitory amino acids in the present experiments do not favour this explanation.

The use of the two-electrode technique in the present investigations enabled administration at sites distant from cell bodies. The value of this technique may provide, as some workers have found, a valuable tool for elucidating the importance to neuronal activation of dendritic receptors (Diamond, 1968; Dudar, 1968; Ransom & Nelson, 1975) and neighbouring neurones (Biscoe & Curtis, 1967; Renaud & Kelly, 1974a, b; Headley, Lodge & Duggan, 1976).

The importance of the present investigation lies in the demonstration of the effects of electrophoretically administered morphine which can be related to analgesia following systemic opiates. Because of this, the technique offers a means of investigating the physiological role, if any, of the polypeptides which compete with morphine for stereospecific receptor sites in brain homogenates (Terenius & Wahlström, 1975).
C. EFFECTS OF OPIATE AGONISTS AND ANTAGONISTS ON NEURONES OF THE MEDIAL THALAMUS

Pharmacological investigations of medial thalamic neurones described here were embarked upon initially in an attempt to determine the activity of opiate compounds in this region. The fact that the medial thalamus had been implicated in the central organization of nociceptive information and mechanisms of analgesia (see later discussion), rendered it a likely site for the interaction of morphine-like substances with central neurones.

Preliminary studies, aimed at characterizing the responses of neurones of the medial thalamus to electrophoretically administered putative transmitter compounds (previously unreported) and to electrical foot-pad stimulation, were carried out. The unexpected actions of acetylcholine in these investigations however, warranted an extended study which has been published (Duggan & Hall, 1975).

(i) INHIBITION OF MEDIAL THALAMIC NEURONES BY ACETYLCHOLINE

Introduction

Early studies of the sensitivity of thalamic neurones to electrophoretically administered substances (Andersen & Curtis, 1964a and b), revealed that acetylcholine excited ventrobasal neurones and the receptors had properties which were intermediate between nicotinic and muscarinic, similar to those of lateral geniculate neurones (Curtis & Davis, 1963) and neurones in the medullary reticular formation (Salmoiraghi & Steiner, 1963). Further studies have
confirmed the pharmacological properties of these thalamic neurones (McCance, Phillis, Tebècis & Westerman, 1968) and along with other reports which suggest the possibility of cholinergic projections to the thalamus (Shute & Lewis, 1963; Austin & Phillis, 1965; Phillis, 1968), provide evidence that acetylcholine may have a function in synaptic transmission processes (see Phillis, 1971).

As pharmacological information, similar to that described above for ventrobasal neurones, has not been reported for the medial thalamic group, the present study was undertaken to define the pharmacological responses of neurones in these regions.

Most animals in these thalamic experiments were anaesthetized with α-chloralose, one animal with pentobarbitone and all were artificially respired. No difference occurred between α-chloralose or barbiturate anaesthetized animals in the type of effects observed.
Fig. 28: A diagramatic representation of some thalamic nuclei in sagital section at the +7 Horsley-Clarke co-ordinate. PF-CM, Parafascicularis-Centre Median Complex; LAT POST, Lateralis Posterior Nucleus; VPM, Ventralis Postero-Medialis; MED GEN, Medial Geniculate; LAT GEN, Lateral Geniculate. A seven barrel micropipette is also illustrated.
RESULTS

Neurones of the centre-median parafascicularis (CM-PF) complex were sought as described in Chapter II. The deposition of dye indicated that neurones in the medial part of nucleus dorsomedialis were also studied. These nuclei are represented diagramatically in Figure 28 although the histological sections did not permit accurate nuclear limits to be defined. Physiological characterization of neurones in terms of nuclei was also not possible and neurones studied responded to peripheral stimulation with latencies of 15-70 msec as previously reported (McKenzie & Rogers, 1973). All cells responded to stimulation of more than one limb. With most neurones, the presence of spontaneous bursts of firing (spindles, Andersen & Sears, 1964) markedly influenced the likelihood of this response to foot pad stimulation; not only did the position of the stimulus in relation to the various phases of the spindle affect the response (Andersen & Sears, 1964), but many neurones could not be activated at all between spindles.

(a) The Effects of Acetylcholine

(i) Spontaneous Firing

Spontaneous activity, commonly in the form of spindling, was exhibited by 63 of 66 neurones studied in the region of the centre median (CM), parafascicularis (PF) and lateralis posterior (LP) nuclei. Acetylcholine, ejected with currents of 35-100 nA, depressed the firing of 41 neurones, excited 10 and had no effect on 15 neurones, three of which had no
Fig. 29: Effects of acetylcholine on spontaneous firing, excitation by L-glutamate and evoked firing of thalamic neurones. A. Depression of spontaneous firing by ejection of acetylcholine 60 nA (ACH), $\gamma$-aminobutyric acid 35 nA (GABA) and acetyl-$\beta$-methylcholine 80 nA (AC $\beta$ ME) and lack of effect by ejection of sodium 80 nA (Na$^+$). B. Depression of spontaneous firing but enhancement of excitation by L-glutamate. C. Depression by acetylcholine, of firing evoked by a stimulus to the ipsilateral forepaw. Each record is a post-stimulus time histogram composed of 64 sweeps with an analysis interval of 5 ms. An arrow marks the position of the stimulus. Stimulus frequency 0.3 Hz. Records B and C are from the same cell.
spontaneous activity.

It was possible to relate the response to acetylcholine to the type of spontaneous activity. Forty-five neurones showed spontaneous spindles and were evoked inconstantly by peripheral stimulation and with a relatively long latency (30-60 msec). Of these, the spontaneous firing of 38 was depressed by acetylcholine, none were excited and that of seven unaffected. In Figure 29A the depression of one neurone's spontaneous spindles by acetylcholine, GABA and acetyl-β-methylcholine and lack of effect by ejection of sodium ions is depicted. Four neurones exhibiting spontaneous spindle activity, but responding constantly to peripheral stimulation and with relatively short latency (12-20 msec), were excited by acetylcholine. Fourteen neurones fired irregularly without spindles and of these, 3 were depressed by acetylcholine, 6 excited and 5 unaffected.

(ii) Excitation by L-glutamate

Since the spontaneous firing rates of the majority of neurones sampled were quite low (e.g. as in Figure 29A), excitation by L-glutamate was initially attempted to demonstrate more clearly the depressant action of acetylcholine. The results were unexpected. Of 17 neurones, the spontaneous firing of which could be depressed by acetylcholine, excitation by L-glutamate was enhanced in 14, depressed in 2 and unaffected in 1.

Excitation by L-glutamate was enhanced concurrently with a depression of spontaneous firing on 14 of 17 neurones, the responses of 2 being depressed and the
remaining neurone being unaffected. This effect was observed in 9 animals using 10 different micropipettes. Figure 29B illustrates on the same cell the depression of spontaneous firing, but the enhancement of the response to L-glutamate, during the ejection of acetylcholine.

(iii) Peripheral Activation

Peri-stimulus histograms, prepared from the responses of spindling neurones to electrical stimulation of the foot pads, revealed both early and late components (Fig. 29C). The early phase had a latency of 20-60 msec and was the firing used to identify neurones. Of 7 neurones analysed in this manner, the early and late evoked firing of 5 was depressed by acetylcholine (40-60 nA) and that of 2 unaffected. Figure 29C shows a 45% decrease in the early firing and near complete abolition of late firing, both effects being reversible. These records were obtained from the same neurone as those of Figure 29B.

(b) Effects of Cholinomimetics

In an attempt to characterize the nature of the depressant effect of acetylcholine, a nicotinic and muscarinic agonist was tested for effects on neurones inhibited by acetylcholine.

Nicotine (30-50 nA) depressed the spontaneous spindle activity of 5 of 6 neurones and acetyl-β-methylcholine (70-90 nA; Fig. 29A) depressed spontaneous spindle activity of 4 neurones tested. The time course of
Fig. 30: The reversal by dihydro-β-erythroidine (DHβE) of the potentiation of L-glutamate (L-GLUT) and suppression of spindles caused by the ejection of acetylcholine. The breaks in the ratemeter record represent periods of 1½ and 4½ minutes respectively.
responses to both these cholinomimetics was similar to that of acetylcholine.

(c) **Effects of Acetylcholine Antagonists**

Dihydro-β-erythroidine (DHβE), a nicotinic antagonist, was ejected with currents of 10-40 nA and reversibly antagonized both effects of acetylcholine, depression of spontaneous spindles (5 of 8 neurones) and enhancement of excitation by L-glutamate (2 of 2 neurones). This action by DHβE is illustrated in Figure 30.

Atropine antagonized depression of spindles by acetylcholine on the two occasions tested.

On no occasion did either of these two substances alter the magnitude of the spindle response or the frequency of spindling.
DISCUSSION

Inhibition of thalamic neurones by acetylcholine, to the extent described in the present study, has not previously been reported. In early investigations of the pharmacological responses of ventrobasal thalamic neurones, excitation by acetylcholine was the predominant effect, although an initial inhibition of several cells was observed (Andersen & Curtis, 1964b). Depression by acetylcholine was an uncommon finding with neurones of the lateral geniculate (Phillis, Tebécis & York, 1967; Satinsky, 1967) and in a survey of several other thalamic nuclei (McCance, Phillis & Westerman, 1968). Tebécis (1970) reported however that 40% of medial geniculate neurones were depressed by acetylcholine and more recently up to 60% of neurones in the anterior pulvinar and nucleus lateralis posterior regions were found to be depressed by this substance (Godfraind, 1975). A recent study of neurones of the feline nucleus reticularis has also reported a considerable proportion of neurones to be inhibited by acetylcholine (Ben Ari, Kanazawa & Kelly, 1976).

The receptor associated with inhibition of medial thalamic neurones in the present study exhibited a mixed nicotinic/muscarinic pharmacology, as has also been found for other receptors mediating inhibition of medial geniculate neurones (Tebécis, 1972) and superficial cortical neurones (Phillis & York, 1968). The most commonly observed type of inhibition mediated by acetylcholine involves receptors of a predominantly muscarinic nature and has been reported in many regions of the CNS (supraoptic nucleus, Barker, Crayton & Nicoll, 1971; cerebral cortex, Randić, Siminoff & Straughan, 1964; Phillis...

In invertebrate central neurones the inhibitory action of acetylcholine is believed to be a postsynaptic effect, the result of which is a change in the chloride conductance of the postsynaptic neurone (Chiarandini & Gerschenfeld, 1967). The mechanism of cholinergic inhibition in the mammalian CNS is yet to be described (see Krnjević, 1974), but the differential effect of acetylcholine on spontaneous firing and on that produced by an excitant amino acid in the present study has not been reported previously and points to a presynaptic action. If it is assumed that excitation by L-glutamate is a postsynaptic action (Curtis, Duggan, Felix, Johnston, Tebėcis & Watkins, 1972), then potentiation of this effect by acetylcholine is most simply explained in terms of acetylcholine also being a postsynaptic excitant, as there is no evidence that cholinomimetics interfere with the uptake of L-glutamate by rat brain slices (Balcar & Johnston, 1972). In view of this effect, a reduction of the spontaneous spindles by acetylcholine is most probably an effect on presynaptic structures, as such firing in ventrobasal and centralis lateralis neurones has been shown to be associated with sequences of excitatory and inhibitory postsynaptic potentials (Andersen & Sears, 1964). The other possible explanation is that acetylcholine blocks the postsynaptic action of the naturally released transmitter which is not L-glutamate.

The "presynaptic" effect of acetylcholine may involve the following mechanisms:
1. Depolarization of nerve terminals. This would result in a decreased output of the natural transmitter as has been described by Hubbard & Willis, (1962) and Eccles, (1964).

2. Excitation of inhibitory neurones. The firing of adjacent neurones which were inhibitory to the neurone under observation would result in a depressant effect being ascribed to acetylcholine, but such an effect alone would be unlikely to differentiate between synaptic and L-glutamate induced firing. A differential effect might arise if the proposed adjacent neurones were on a pathway for presynaptic inhibition. L-glutamate, however, excites neurones widely in the mammalian central nervous system (Curtis et al., 1972) and failure to observe similar inhibition with this substance implies that there are marked differences between the cells studied and the proposed adjacent inhibitory neurones in their sensitivity to acetylcholine.

3. Inhibition of adjacent neurones which were excitatory to the cells studied. Again this mechanism would fail to produce a differential effect by acetylcholine on the synaptic and L-glutamate induced firing.

There is evidence that acetylcholine has a presynaptic depolarizing action at the mammalian neuromuscular junction (Riker & Okamoto, 1969), frog sympathetic ganglia (Riker, 1968) and at central molluscan neurones (Woodson, Schlapfer, Tremblay & Barondes, 1975). At all of these synapses, where acetylcholine is believed to be the transmitter mediating postsynaptic excitation, the consequence of this presynaptic action is an impairment of transmission.
The finding in the present study of an inhibitory action of acetylcholine on medial thalamic neurones is of interest when considered with other studies where the predominant effect of this ester and possible transmitter is reported to be excitation (Andersen & Curtis, 1964a). The possible functional significance of this finding requires further experimentation. Firing in spindles by thalamic neurones is not seen in conscious animals but is associated with slow wave sleep and anaesthesia (Filion, Lamarre & Cordeau, 1971). Acetylcholine, along with its synthetic and degradative enzymes, has been shown to be present in the thalamus (MacIntosh, 1941; Feldberg & Vogt, 1948; Zetler & Schlosser, 1955; Hebb & Silver, 1956; Burgen & Chipman, 1951, 1952; Koelle, 1954; Gerebtzoff, 1959), however it remains to be shown that a functional role of this putative transmitter, possibly as part of the "ascending activating system" (see Brodal, 1969), is related to its suppression of medial thalamic neurones.
(ii) ACTIONS OF OPIATE AGONISTS AND ANTAGONISTS ON MEDIAL THALAMIC NEURONES

Introduction

Although a previous section of this thesis reports evidence favouring a role for the spinal cord in the mechanism of morphine analgesia, other brain regions are probably affected by systemic opiates (see Concluding Remarks).

The medial thalamus is one such area and on both anatomical and physiological grounds is believed important in the appreciation of nociceptive information. These nuclei have been shown by degeneration studies to receive spinothalamic extralemniscal projections (cat, Anderson & Berry, 1959; Boivie, 1971; monkey, Mehler, et al. 1960; Bowsher, 1961). There is some doubt over the existence of direct spinal projections to the centre-median nucleus (see Kerr & Lipman, 1974) and the possibility that spinothalamic projections to this nucleus are polysynaptic via bulbar and pontine nuclei is most likely (see Bowsher, 1966). Additional support for these anatomical findings comes from reports of diminished sensitivity to pain which follows lesions of this area (in particular the centre-median, parafascicularis complex) both in animals (Mitchell & Kaelber, 1966; Marburg, 1973; Kaelber, Mitchell, Yarmat, Afife & Lorens, 1975) and man (Hecaen, Talairach, David & Dell, 1949; Mark & Ervin, 1969). Chang (1973) has also suggested from electrophysiological studies a role of these nuclei in the analgesia resulting from acupuncture.
On physiological grounds, the medial thalamus has also been suggested to be important in nociception. Albe-Fessard & Kruger (1962) recording from single units in the centre-median, demonstrated that "noxious" stimuli and not those produced by hair bending, light touch or muscle stretch excited these neurones. Similarly Woda, Azerad, Guilbaud & Besson (1975) were able to elicit responses of single neurones of the medial thalamic nuclei by stimulation of the dental pulp of the cat, a procedure believed to elicit a purely painful sensation (Windle, 1927; Brookhart, Livingston & Haugen (1953).

It is not surprising that such an area has attracted interest as a site where morphine-like drugs might interact with central neurones in the production of analgesia. Indeed evidence supporting such a possibility has accumulated from micro-application experiments (Yeung, Yaksh & Rudy, 1975), and opiate binding studies (Kuhar, Pert & Snyder, 1973). Differences however, have been observed between morphine and meperidine in their effects on gross potentials recorded in the centre-median (Millan & Besson, 1964).

The present investigations examined the effects of morphine and naloxone on the firing patterns of single neurones of the centre-median (CM), parafascicularis (PF) and lateralis posterior (LP) nuclei when administered both electrophoretically and intravenously. Animals were anaesthetized with α-chloralose and were artificially respired.
RESULTS

(a) Morphine and Naloxone Administered Electrophoretically

(i) Spontaneous Firing

The effects of morphine were studied on 32 cells which fired spontaneously with a spindle pattern. Ejected with currents of 20-100 nA, morphine had no effect on the firing of 18 neurones, weakly depressed that of 13 and excited 1 (Fig. 31B). In no case did the available currents produce greater than 75% inhibition of firing, and by comparison, relatively low currents of acetylcholine (Fig. 31A, B) and GABA were more potent depressants.

Naloxone (15-100 nA), weakly depressed the spontaneous firing of 7 neurones (Fig. 31A) and had no effect on that of 18. Owing to the weak and inconsistent depression by morphine, on only 6 occasions could quantitative tests of possible antagonism by naloxone be performed and in no case was this observed.

(ii) Firing Evoked by Electrical Stimulation of Forepaws

To assess the effects of electrophoretically administered morphine and naloxone on evoked firing, peri-stimulus time histograms were prepared. The more medially located cells of the centre-median parafascicularis complex responded to stimuli to the forepaws with a relatively short latency (15-20 ms) and constantly. Cells of the nucleus lateralis posterior region fired with a longer latency (20-80 ms) and the presence of spindles markedly influenced the probability of a response, as reported in the previous
Fig. 31: Effects of electrophoretic morphine and naloxone on spontaneous and chemically induced firing of medial thalamic neurones. Each trace is a pen recording of cell firing measured with a rate meter. **Ordinates:** firing frequency in spikes per second. **Abscissae:** time in minutes. The bars indicate the times of ejection of compounds electrophoretically. A. Depression of spontaneous firing by acetylcholine 25 nA (ACH), morphine 70 and 100 nA and naloxone 80 nA. B. Enhanced excitation by L-glutamate following ejection of morphine. Three segments of cell firing are illustrated being, from left to right, control responses to ejection of L-glutamate 60 nA (L-GLUT), responses during and after ejection of morphine 50 nA, and responses 9 minutes later. Morphine was ejected for a total of 8 minutes only one minute of which is illustrated. C. Enhanced excitation by L-glutamate following ejection of naloxone. Naloxone was ejected for a total of 6 minutes, not all of which is illustrated.
section. Histograms were prepared from both types of cell with similar results.

Morphine (30-80 nA) significantly reduced the evoked firing of 9 cells and had no effect on that of 3. With 7 of the cells depressed by morphine, possible antagonism by electrophoretic naloxone was tested but observed in only 1 instance; in all others naloxone was a depressant of firing at least as potent as morphine, and the effects were additive and not antagonistic.

(iii) Responses to Acetylcholine

Morphine potentiates the excitation of ventro-basal thalamic neurones by acetylcholine, an effect antagonized by naloxone (Duggan, Davies & Hall, 1976). A similar action by morphine on medial thalamic neurones might be expected to result in a diminished responsiveness to peripheral stimuli as these cells are inhibited by acetylcholine (preceding section; Duggan & Hall, 1975).

Very little, if any, interaction between acetylcholine, morphine and naloxone was observed. Naloxone reversed the depression of firing by acetylcholine on only 2 of 15 neurones; and morphine enhanced depression by acetylcholine on 1 of 8 neurones tested. Two neurones were excited by acetylcholine and morphine enhanced this effect.

(iv) Excitation by L-Glutamate

In contrast to the weak effects on spontaneous and evoked firing, interactions of electrophoretically administered morphine with excitation by L-glutamate,
when present, were marked. Of 18 neurones tested, excitation by L-glutamate, together with spontaneous firing, was depressed in 6 cases, enhanced in 2 and unaffected in 10, during ejection of morphine (30-80 nA). Immediately following the period of morphine ejection however, a large (up to 300%) and long lasting (8-43 min) increase in L-glutamate sensitivity occurred with the 8 cells, the responses of which had been altered during morphine ejection. Results from one are illustrated in Figure 31B. The duration of this effect was not determined with any accuracy as with 6 neurones the L-glutamate response was still enhanced when recording conditions did not permit accurate counting of neuronal firing rates.

As this enhanced sensitivity to L-glutamate occurred after and not during morphine ejection, the following experiments were performed to investigate the possible relevance of this phenomenon to the syndrome produced in humans after withdrawal of morphine.

1. In the dependent animal, withdrawal symptoms can be produced by administration of a narcotic antagonist (Martin, 1967). In the present experiments when the responses of cells to L-glutamate had been depressed by morphine, the added ejection of naloxone did not enhance the sensitivity to L-glutamate. These substances were additive as depressants of L-glutamate evoked firing.

2. Subsequent doses of morphine during the
Fig. 32: The positions marked with filled circles indicate, on the outline of a brain section at Horsley-Clarke +7 co-ordinates, the location of 14 neurones on which studies of the effects of intravenous morphine and naloxone were carried out. The abbreviated anatomical features are HIP TR habenular interpeduncular tract; MB mammillary bodies; LG lateral geniculate.
period of enhanced L-glutamate sensitivity produced an immediate depression of activity. Similar currents of naloxone, however, also depressed the enhanced L-glutamate responses. Ejection of sodium ions was without effect thus excluding the possibility that the effects observed were due to current alone. Enhanced sensitivity to L-glutamate, following ejection of morphine was also observed with naloxone. Of 12 neurones tested, the responses of 4 to L-glutamate were reduced by ejection of naloxone and with 3 of these long lasting enhanced sensitivity to L-glutamate was observed following the period of naloxone ejection (Fig. 31C).

The ejection of GABA with currents adequate to produce depression both of L-glutamate firing and spontaneous spindles was not followed by enhanced sensitivity to L-glutamate.

(b) Morphine and Naloxone Administered Intravenously

The present study investigated the effects of intravenously administered morphine and naloxone on the firing patterns of medial thalamic neurones, the locations of 14 of which are shown in Figure 32. Three types of firing pattern were studied:

(i) Spontaneous Firing

Twelve cells fired spontaneously with a spindle pattern, and neither intravenous morphine nor subsequently administered naloxone had any significant effect on such firing in ten experiments (Fig. 33).
In two experiments morphine (0.5 mg kg\(^{-1}\)) disrupted the spindle pattern resulting in increased irregular spontaneous firing, an effect reversed by naloxone.

(ii) Firing Evoked by Electrical Stimulation of Forepaws

As discussed previously, the responses of cells of this region of the thalamus vary considerably in constancy and latency. Three neurones located medial to the habenulo-interpeduncular tract responded constantly to the stimulus with a latency of 13-15 ms. Intravenous morphine (1.5 mg kg\(^{-1}\) total) significantly reduced the responses of one cell but had no effect on the remaining two. Naloxone (0.3 mg kg\(^{-1}\)) administered from 9 to 35 minutes after morphine not only reversed the depression of evoked responses by morphine but enhanced them above control levels. Four cells, more laterally situated, responded with a longer latency and the probability of a response was influenced by the presence of spindle-like firing. In no case did morphine (1.5-2.5 mg kg\(^{-1}\)) influence the total number of evoked action potentials, but with one neurone the latency of activation increased from 65 to 90 ms following morphine 1.0 mg kg\(^{-1}\), and this was reversed by naloxone.

(iii) Excitation by L-glutamate

This series of experiments was prompted by the long lasting increase in L-glutamate sensitivity observed following the electrophoretic administration of morphine.

In 7 of 10 experiments the sensitivity of medial thalamic neurones to L-glutamate was reduced by a
Fig. 33: Depression of sensitivity to L-glutamate by intravenous morphine and its reversal by naloxone. The times of electrophoretic ejection of L-glutamate (60 nA) and γ-aminobutyric acid (GABA) 20 nA are indicated by the continuous and interrupted bars respectively. Fifteen minutes elapsed between the taking of the upper and lower records. Note the change in the ordinate scales between the two records. The last illustrated response to L-glutamate exceeded the full deflection of the pen recorder.
dose of morphine as low as 0.5 mg kg\(^{-1}\), and was completely restored or enhanced above control levels by subsequent naloxone (0.2-0.5 mg kg\(^{-1}\)). To ensure that such changes were not produced merely by alterations of the distance between the micropipette and the cell, as a consequence of the circulatory effects of morphine, in 9 experiments the ejection of L-glutamate was alternated with that of GABA, but no reversible effect on depression of spontaneous firing by GABA was observed following morphine and naloxone. Furthermore, the onset of changes in blood pressure did not correlate with changes in sensitivity to L-glutamate. Results from one experiment are shown in Figure 33. The sensitivity of this neurone to GABA gradually increased throughout the period illustrated, in contrast to that to L-glutamate following morphine and naloxone. This observation excludes the possibility that the circulatory effects of morphine and naloxone produced artifactual changes in the sensitivity to L-glutamate. The changes in sensitivity to L-glutamate occurred within four minutes of injecting morphine and naloxone in all but one experiment. In this instance, two doses of naloxone (0.3 and 0.2 mg kg\(^{-1}\)) were given at an 8 minute interval, and the sensitivity to L-glutamate returned 5 minutes after the second dose.

The observed reduction in sensitivity to L-glutamate occurred in the absence of any significant change in spontaneous spindle-type firing in nine experiments. In the tenth the spindles were disrupted and the mean
spontaneous firing rate was increased. In only one instance was synaptic activation (by foot pad stimulation) concurrently studied owing to the requirement that reproducible electrophoretic administrations of a compound require it to be ejected for fixed times and at fixed intervals. Synaptic activation in this case was not significantly altered despite a reduction in L-glutamate sensitivity.
DISCUSSION

The present study shows that morphine when administered electrophoretically is a weak depressant of neurones of the centre-median, parafascicularis and lateralis posterior nuclei. The finding however, that naloxone similarly administered has the same action or is without effect, renders unlikely the possibility that this observed effect of morphine is relevant to its effect when administered systemically. The possible limitation of the electrophoretic technique in such situations will be discussed subsequently.

From investigations where interactions of morphine or naloxone with acetylcholine were tested, evidence for agonist and antagonist activity at presynaptic receptors for acetylcholine was not obtained. The finding in the previous section, that these cholinergic receptors are mixed nicotinic-muscarinic in nature, whereas previous evidence (Chapter III) has shown that the cholinergic receptors where morphine and naloxone exert agonist and antagonist actions respectively are those possessing predominantly nicotinic properties and which are postsynaptic, is not sufficient explanation for the present results. The former type of receptor is also present on ventrobasal thalamic neurones, the studies of which in this laboratory have revealed similar effects of morphine and naloxone as on Renshaw cells (Duggan, Davies & Hall, 1976).

The increase in sensitivity to L-glutamate following morphine ejection has been observed before in a study of cerebral cortical neurones (Bioulac, Lund & Puil, 1975), but not for the duration observed in the present study. A simple explanation for this long lasting elevated sensitivity to
L-glutamate is that morphine is able to exert a dual action - short lasting depression and long lasting increase in cell excitability. An alternative explanation, that morphine brings about a reduced inactivation of L-glutamate, presumably by inhibition of uptake processes would also explain the prolonged enhanced sensitivity to this amino acid. In vitro studies however, have shown no effect by morphine on these processes (Balcar & Johnston, 1972).

Frederickson, Norris, Hewes (1975) have recently provided, from studies in the rat, the only other report of the effects of an electrophoretically administered opiate compound on medial thalamic neurones. They reported that naloxone was able to both excite and depress these neurones. This work is difficult to compare with the present results: different species were used and spontaneously active and not synaptically activated neurones were sampled.

The significant finding of the present investigations is the reduction by intravenous morphine of the sensitivity of medial thalamic neurones to L-glutamate and its reversal by naloxone. Depression of cell excitability by morphine is the simplest explanation for its effect, this being brought about by a direct action of morphine on the cell membrane or indirectly via inhibitory synaptic influences from other neurones. The former effect could be produced by a direct effect on membrane conductance or a local anaesthetic effect, a property that has been demonstrated with high concentrations of morphine-like compounds (Simon & Rosenberg, 1970) but which is neither stereospecific (Seeman, Chou Wong & Moyyen, 1972) nor reversed by antagonists (Simon & Rosenberg, 1970). The reduction of cell excitability, however, whether a consequence
of this property of morphine or of inhibitory synaptic
influences from adjacent neurones, could be expected to
reduce spontaneous firing and this was not observed. It
is possible that the spindle type of spontaneous firing,
when compared to excitation by electrophoretically adminis-
tered L-glutamate, is an insensitive gauge of cell firing
because it is the result of intense excitatory and inhibitory
postsynaptic potentials (Andersen & Sears, 1964). The find-
ing, that GABA administered electrophoretically reduces both
of these types of firing in parallel however, renders this
possibility unlikely. These considerations lead to the pro-
posal that morphine directly depressed the sensitivity of a
proportion of neurones studied to L-glutamate, independent of
any change in basal excitability.

The finding that the reduction in sensitivity to
L-glutamate by morphine administered electrophoretically was
not antagonized by similarly administered naloxone is diffi-
cult to reconcile with results obtained employing the intra-
venous route of administration. The most likely explanation
which accounts for the results reported here is that
L-glutamate and the morphine-like compounds, when administered
from micro-pipettes, differ considerably in diffusion through
nervous tissue and fail to attain the distribution appropriate
to that achieved when morphine is administered intravenously.
Another possible explanation, which is suggested by previous
findings, is that microelectrophoretic administration failed
to achieve adequate concentrations of morphine at sites,
perhaps dendritic, distant from cell bodies. A detailed
study of the cytoarchitecture of neurones of this region
(Scheibel & Scheibel, 1966) has shown that the dendritic
processes radiate from the cell body, are poorly ramified and of varying length.

Investigations on spinal neurones reported previously in this thesis, and together with those by Dostrovsky & Pomeranz (1973), Calvillo, Henry & Neumann (1974) and Zieglgänsberger, Satoh & Bayerl (1975), have also shown the ability of electrophoretically administered morphine to reduce excitation by L-glutamate. The results reported previously here have shown that this effect by morphine could not be reversed by naloxone. Some workers have reported such antagonism (Calvillo et al., 1974; Zieglgänsberger et al., 1975). In addition Calvillo et al. (1974) in a preliminary communication reported that morphine depressed the sensitivity to L-glutamate only of those neurones which responded to noxious stimulation, and that intravenous naloxone reversed this effect on 2 of 4 occasions, while electrophoretically administered naloxone reversed this effect on only 2 of 7 neurones. (See Chapter III (iii)).

The literature regarding the interaction between opiates and amino acid putative transmitters such as L-glutamate is relatively brief. There is neurochemical evidence (Sherman & Gebhart, 1974) that in mice, noxious stimulation, exposure to a hot plate, decreases the level of L-glutamate found in the peri-aqueductal grey region of mice, and that morphine prevents this change in a manner which is antagonized by prior administration of naloxone.

In conclusion then, the present investigations of medial thalamic neurones have shown that of the three types of firing pattern studied (spontaneous, and that produced by electrical stimulation of the forepaws or by L-glutamate),
only that produced by electrophoretic administration of L-glutamate was found to be altered by low doses of intravenous morphine. The finding, that what might be anticipated as more conventionally tested patterns of neuronal activity were not susceptible to morphine, was unexpected, and it may be that the firing induced by L-glutamate is a more sensitive test of cell excitability. Further investigations might clarify the mechanism by which morphine selectively depresses the action of this substance, and whether this effect results in the depression of a particular neuronal or fibre population, perhaps those involved in the central organization of information in nociceptive pathways.
CHAPTER IV - THE USE OF SNAKE NEUROTOXINS FOR IDENTIFICATION AND CHARACTERIZATION OF ACETYLCHOLINE RECEPTORS IN THE CENTRAL NERVOUS SYSTEM - A PHARMACOLOGICAL STUDY

Introduction

Since the isolation and characterization of α-bungarotoxin (Chang & Lee, 1963; Mebs, Narita, Iwanaga Samejima & Lee, 1971) and cobra neurotoxin (Lee et al., 1968; Karlson, Arnberg & Eaker, 1971) these polypeptides have been widely used for their ability to bind to post-synaptic receptors for acetylcholine (Lee, Tseng & Chiu, 1967). The types of studies, utilizing the essentially irreversible binding properties of these purified snake toxins, include investigations of neuromuscular transmission (Chang & Lee, 1963; Miledi & Potter, 1971) and attempts to localize, isolate and characterize the receptor for acetylcholine in various tissues (Changeaux, Kasai & Lee, 1970; Miledi, Molinoff & Potter, 1971).

Although α-bungarotoxin and cobra neurotoxin do not pass the blood-brain barrier, and therefore do not enter the central nervous system to a measurable extent (Lee & Tseng, 1966), several investigators have used these toxins in attempts to characterize, in vitro, acetylcholine receptors in the vertebrate brain (Salvaterra & Moore, 1973; Schleifer & Edelfrawi, 1974; Eterovic & Bennett, 1974; Polz-Tejera, Schmidt & Karten, 1975). Although binding activity of these toxins to brain preparations is highest in synaptosomal fractions (Salvaterra & Moore, 1973), and is inhibited by various
nicotinic compounds (Eterovic & Bennett, 1974), the significance of these findings is uncertain in the absence of knowledge of the effects of toxin on central nicotinic receptors in vivo.

In invertebrate preparations, α-bungarotoxin has been shown to antagonize the response to acetylcholine of these three types of central molluscan cholinergic receptors (Shain et al., 1974) but two subsequent reports (Szczepaniak, 1974; Kehoe et al., 1976) found however, that α-bungarotoxin antagonized only a cholinergic inhibitory mechanism which was chloride dependent.

In the cat, the nicotinic receptors of sympathetic ganglia are unaffected by locally injected cobra neurotoxin (Chou & Lee, 1969) or α-bungarotoxin (Lee & Lee, unpublished). Obata (1974), working with cultured sympathetic ganglia and atrial muscle cells of the rat, demonstrated no antagonism of the responses to acetylcholine by α-bungarotoxin. In addition, the dorsal root potentials produced by stimulation of the ventral roots of the isolated frog spinal cord, a mechanism which involves a nicotinic cholinergic synapse (Kiraly & Phillis, 1971) are not reduced by high concentrations of α-bungarotoxin (Miledi & Szczepaniak, 1975).

In an attempt to resolve whether these neurotoxins were active at central receptors for acetylcholine in the cat, α-bungarotoxin (αBTX) and cobra neurotoxin (CTX) were administered from micropipettes in the vicinity of Renshaw cells. The synaptic activation of these neurones and their excitatory responses to acetylcholine and excitant amino acids administered from micropipettes were studied. Both α-bungarotoxin and cobra neurotoxin were administered by
several different procedures as described in Methods
and all animals were anaesthetized with pentobarbitone sodium.
### TABLE VIII - EFFECTS OF COBRA NEUROTOXIN AND α-BUNGAROTOXIN ON THE CHOLINERGIC ACTIVATION CHEMICAL SENSITIVITY OF RENSHAW CELLS

<table>
<thead>
<tr>
<th>Toxin and method of administration</th>
<th>Cholinergic Activation</th>
<th>Chemical Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No effect</td>
<td>Reduced</td>
</tr>
<tr>
<td>α-bungarotoxin:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>electrophoresis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pressure</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cobra neurotoxin:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pressure - parallel assemblies</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>pressure - 5 barrel electrode</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>local injection</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td>14</td>
<td>1</td>
</tr>
</tbody>
</table>
RESULTS

The effects of the neurotoxins, administered both electrophoretically and by pressure, on excitation by electrophoretically administered excitants, were studied on 45 Renshaw cells. With 16 of these, cholinergic synaptic activation was studied concurrently and with 3 neurones, synaptic activation alone was measured. The results are summarized in Table VIII.

Of the seven cells in which a reduction in sensitivity to electrophoretic acetylcholine occurred, cholinergic synaptic activation was studied in 5 and a reduction observed in only one. No reduction in synaptic activation was observed with the two cells in which sensitivity to L-glutamate declined following ejection of toxin. Recovery studies were incomplete, but with two cells in which α-bungarotoxin ejection was associated with a reduction in acetylcholine sensitivity, recovery was observed at 5 and 118 min, and with another no recovery was present 14 min after cessation of ejection.

Under the experimental conditions used, ejection of the two snake neurotoxins had no significant effect in reducing cholinergic synaptic activation of Renshaw cells. Indeed an enhanced response was observed with 4 of 19 neurones. This probably resulted from the mechanical disturbances of ejection by pressure, as an increase in spontaneous firing was observed with all four cells immediately on application of pressure.

No significant effect on chemical sensitivity was observed following ejection of the neurotoxins. While a reduction in the response to an electrophoretic excitant...
occurred more commonly with acetylcholine than with L-glutamate, failure to observe a concurrent reduction in synaptic activation makes it probable that changing cell-pipette distances and not blockade of acetylcholine receptors was responsible for this result. Concentrations of dihydro-β-erythroidine ejected by pressure and adequate to reduce the response to acetylcholine, also reduced ventral root evoked firing. The problems of quantitative measurements of chemical sensitivity outlined in Methods do not apply to synaptic activation and it was clear that this was unaffected by ejection of the neurotoxins.

To ensure that the lack of effects obtained with αBTX and CTX was not due to failure to spread from the site of pressure injection to surrounding areas of the nervous tissue, the distribution of radiolabelled αBTX and CTX, when ejected in the same manner, was subsequently studied. A similar study (Polz-Tejera, Schmidt & Karten, 1975) but using in vitro methods has reported that certain brain regions "bind" radiolabelled α-bungarotoxin better than others, but the precise cellular localization of binding was unable to be determined.

The potency of the α-bungarotoxin following the iodination procedure was checked and the sample caused death within 15 minutes of an I.P. injection of 0.60 μg/g of αBTX to a mouse (4LD50; Lee et al., 1972). Insufficient radiolabelled cobra neurotoxin was available for this test of potency.

Autoradiographic analysis of the 125I-labelled compounds at various times after ejection showed that they diffused readily in the spinal cord. Even at 3½ hours after injection, labelled toxin had extended to the edge of the largest block
Fig. 34: A. Labelling in the vicinity of end plates of the diaphragm of a mouse killed by injection of radiolabelled α-bungarotoxin. B. Labelling of neurones in the lumbar spinal cord adjacent to an injection site of cobra-neurotoxin; seven hours between injection and fixation. C. Labelling of blood vessel walls but not spinal neurones following injection of α-bungarotoxin 3½ hours previously. The section was 2.1 mm from the injection site. D. Labelling of blood vessel walls but not spinal neurones following injection of cobra-neurotoxin 4 hours previously. The section was 1.3 mm from the injection site. B & C from medial ventral horn; D, from lateral ventral horn. Calibration bar: 50 μm.
of tissue i.e. greater than 2.2 mm from the injection site. As the sections were 5 µ thick, the cell membranes of most spinal neurones would be seen in outline. If extracellular binding had occurred the resultant appearance could be expected to be an accumulation of silver grains outlining the body. Areas of cell body entirely within the plane of section, either cells cut obliquely or cell processes, would be uniformly labelled. As Renshaw cells have relatively small somata (10-15 µ; Jankowska & Lindstrom, 1971), uniform labelling would be commonly expected.

The diaphragm of the mouse used above to test the toxins' potency was found by autoradiographic examination to be highly labelled (Fig. 34A).

In spinal tissue sections, accumulations of silver grains were observed over two types of structures - neurones (Fig. 34B) and blood vessel walls (Fig. 34C, D). The latter were commonly labelled, presumably as a result of the difficulty with which toxins penetrate capillary walls to the lumen.

Neurones on the other hand were labelled only in the vicinity of the injection sites and to a similar extent in both dorsal and ventral horns; no neuronal accumulations of grains were observed more than approximately 150 µ from the injection site.
DISCUSSION

From the results of the autoradiographic study, it is unlikely that the lack of effects of ejected toxins on Renshaw cell firing in response to synaptic and chemical activation was due to failure of spread from the injection site. Labelled αBTX has also been shown to penetrate readily the isolated frog spinal cord (Miledi & Szczepaniak, 1975). Studies in which proteins have been ejected from micro-pipettes are few, but it has been estimated from effects on adjacent neurones, that tetanus toxin injected into the cerebellar cortex of the cat from a pipette of tip diameter 25 μm, spreads at a rate of 0.5-1.0 mm/hr (Curtis, Felix, Game & McCulloch, 1973). A similar rate of distribution has been found by the present investigations.

Although similarities have been noted between the effects on the rat cerebral cortex of locally applied strychnine, curare and a cobra neurotoxin fraction (Bhargava, Horton & Meldrum, 1970), in the cat the toxicity of intracisternal whole cobra venom has been attributed to cardiotoxin and phospholipase A and not to neurotoxin (Lee, 1971). A similar deduction has been made for the effects of cobra venom on ganglionic transmission in the cat (Chou & Lee, 1969). It has been proposed that the acetylcholine receptors of Renshaw cells have properties more related to those of sympathetic ganglia than to skeletal muscle (Curtis & Ryall, 1966), a suggestion consistent with the failure of αBTX and CTX to block activation of Renshaw cells and ganglion cells.

The labelling of neurones in both dorsal and ventral horns and within only a very small distance from the
injection sites, implies that it was a consequence of severance of neuronal processes by the penetrating micropipettes, with a resultant intracellular accumulation of toxin: the uniform labelling of these cells, which could be observed in several serial sections, indicates an intracellular location of the toxin.

It is unlikely that the failure to observe silver grain accumulations indicating extracellular binding was due to the relatively high background, since such accumulations were readily detectable around blood vessels (Fig. 34C, D). Moreover, if the receptor density on the surface of neurones receiving a nicotinic innervation is comparable to that at mouse diaphragm end plates (Fig. 34A), then the grain density resulting from binding of labelled toxin should have been sufficiently above background to permit detection. The amounts of toxin expelled should have been adequate to saturate nicotinic receptors for a considerable distance from the injection site. In the case of αBTX, approximately 0.46 μg was expelled at each site whereas 12.0 μg in the whole mouse (20g) produced heavy labelling of end plates.

The significance of the binding of these toxins to brain slices (Polz-Tejera, Schmidt & Karten, 1975) and homogenates (Eterovic & Bennett, 1974) is uncertain when considered with the present results. Intracellular binding of αBTX (Porter, Chui, Weikowski & Barnard, 1973) and of CTX (Libelius, 1975) has been demonstrated with mouse skeletal muscle and such sites are widely distributed and not related to end plates. Intracellular binding of [14C]nicotine has been demonstrated in rat brain slices (Alderice & Weiss, 1975). It is perhaps this binding which was observed when thin slices were
incubated with αBTX.

These results from autoradiographic studies support those obtained from extracellular recording studies with Renshaw cells and together with other reports (Obata, 1974; Miledi & Szczepaniak, 1975; Lee & Lee, unpublished) render it unlikely that these toxins are useful markers for the presence, distribution and characterization of nicotinic receptors within the vertebrate central nervous system.
CHAPTER V - CONCLUDING REMARKS

The results presented in this Thesis have provided evidence for an interaction of opiates, both at receptors for acetylcholine and at sites of unknown identity in the dorsal horn of the spinal cord. The apparent diversity of these findings warrants some concluding remarks.

The studies with Renshaw cells suggested that opiates may produce some of their effects by an action at nicotinic receptors for acetylcholine in the central nervous system. This possibility has been supported by the finding of similar effects of morphine at the mixed cholinergic receptors of ventro-basal thalamic neurones but not at those of the muscarinic receptors of the cerebral cortex (Duggan, Davies & Hall, 1976).

That naloxone administered electrophoretically reversed the excitation produced by similarly administered morphine at nicotinic receptors, was taken as evidence of the pharmacological specificity of morphine's effect and therefore of its possible relevance to systemic effects of opiates. These results were supported by studies with morphinan compounds, although significant differences between the effects of these and of morphine and naloxone could be observed. Studies with physostigmine failed to define the mechanism of action of the effect of morphine although a postsynaptic site was implicated. The results obtained with oxymorphone were not altogether consistent with an action of opiates at nicotinic receptors.

The later results, which demonstrated that morphine administered in the region of the substantia gelatinosa,
but not near cell bodies, produced a selective depression of the response of dorsal horn neurones to noxious stimulation, have been interpreted as a relevant effect of this opiate, not only because of its selectivity in the modality affected but also because of its reversibility by similarly administered naloxone. The important claim to relevance of these later experiments is not reversibility by electrophoretic naloxone but the finding that low doses of intravenous naloxone could produce reversal of the effect of morphine.

It is conceivable that an effect of a compound, when administered electrophoretically, becomes evident merely as a result of high enough concentrations of that compound at appropriate receptors. It is as equally conceivable that an antagonist, similarly administered, and in high concentrations, might antagonize such effects at the same receptors. This possibility may apply to the effects seen at nicotinic receptors of Renshaw cells in the initial studies. Therefore, it is desirable, for the argument of physiological relevance, to show that effects observed with one compound are reversible with low intravenous doses of its antagonist. Such a demonstration was possible with the studies where morphine was administered in the substantia gelatinosa region.

There is a vast literature concerning the site of action of morphine-like compounds and convincing evidence has been provided for an action both at the spinal level and at higher brain centres (spinal cord literature already discussed; Liebeskind, et al., 1974; Dey & Feldberg, 1975). Clearly both sites are important and a modulating role by higher centres of spinal cord processes has been suggested (Satoh
& Takagi, 1971; Heavner, 1975). The results from the present study, with administration to the substantia gelatinosa, suggest the importance of a spinal action of opiates.
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