NEUROPEPTIDES AND SPINAL NOCICEPTIVE MECHANISMS

BY

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Most of the experiments described in this thesis were performed in collaboration with scientific colleagues.

I was the principal investigator responsible for planning and carrying out those experiments dealing with:

(a) The physiology and pharmacology of segmental inhibition of the spinal transmission of nociceptive information;
(b) D-Phenylalanine as an inhibitor of enkephalin degradation;
(c) The pharmacology of noradrenergic inhibition in the spinal cord and the possible involvement of noradrenaline in inhibition resulting from electrical stimulation in the dorsolateral pons;
(d) The actions of a novel endogenous opioid peptide, metorphamide, administered near spinal neurones.

The other experiments on opioid peptide physiology and pharmacology were performed with in collaboration with Drs A.W.Duggan, C.R.Morton and J.G.Hall. The studies on substance P release were performed with Drs A.W.Duggan and I.A.Hendry.

Zhi-Qi Zhao
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CHAPTER 1 GENERAL INTRODUCTION

The aim of this thesis was to investigate the mechanisms involved in the actions of neuropeptides on the transmission of nociceptive information in the spinal cord of the cat, and the segmental and descending inhibitions which control the firing of dorsal horn neurones.

In the last decade there has been an exponential enrichment in our knowledge of the physiology and pharmacology of pain, principally due to the discovery of a large number of neuroactive peptides in the CNS. Nociception can be substantially modulated by many peptides. Of these peptides substance P (SP) and the opioid peptides have been studied extensively. SP was proposed to be a transmitter released from the central terminals of nociceptive primary sensory neurones. Opioid peptides depress transmission of nociceptive information in the spinal cord and are likely to be mediators of antinociceptive actions. However, the mechanisms underlying these physiological processes are still unclear.

The experiments described in this thesis are summarized below: (i) To study whether substance P is released from the central terminals of nociceptive afferent fibres; (ii) To study the actions of opioid peptides and peptidase inhibitors on the transmission of nociceptive information by dorsal horn neurones; (iii) To study segmental and descending inhibitions influencing spinal nociception and the possible transmitters relevant to such inhibition.
2.1. Animals and Surgery

Experiments were conducted on cats weighing 2.0 to 3.5 kg anaesthetized with pentobarbitone sodium (35 mg/kg i.p. initially). All animals were artificially ventilated after neuromuscular paralysis with gallamine triethiodide (4 mg/kg, initial i.v dose) and end-tidal CO$_2$ level kept at 4 %. A slow infusion pump delivered both pentobarbitone (2 mg/kg/h) and gallamine (4 mg/kg/h) continuously throughout the recording period. Blood pressure was monitored with a cannula in a common carotid artery and experiments terminated if systolic pressure (in the absence of cold block of spinal conduction, or electrocoagulation in the brainstem) was less than 100 mmHg. Body temperature was automatically maintained at 37°C by heating pads placed under the animal body.

2.1.1 Peripheral nerve

In most investigations of the nociceptive responses of dorsal horn neurones, the tibial nerve was exposed for stimulation. In some experiments the plantar nerve was exposed for stimulation and a small filament separated for recording. For experiments concerned with spinal reflexes and motoneurones, the nerve to the post biceps-semiendinosus (PBST), nerves to the medial and lateral gastrocnemius muscles (ML-G), the sural nerve and the tibial nerve were exposed and sectioned distally. All of these nerves were
immersed in a pool of warm paraffin oil.

2.1.2. Spinal cord

The lumbar spinal cord was exposed by laminectomy. The dural sac was carefully opened dorsally over the lumbar-sacral cord. The exposed cord, lying in an approximately horizontal plane, was covered by warmed paraffin oil. In experiments using antibody microprobes a layer of Ringer-agar was used instead of paraffin oil. In some experiments, three ventral roots (L₆, L₇ and S₁) were divided distally and mounted on platinum electrodes.

**Cold block (Chapter 5):** In some experiments spinal conduction was reversibly blocked by cooling at the L₁ level. A small metal chamber with a concave lower surface closely contacted the dorsal surface of the first lumbar segment of the cord over a distance of approximate 1 cm and a silicone grease was applied along the contact edges of the chamber with the spinal cord. The temperature of the spinal cord surface was monitored by a thermistor positioned in the base of the chamber. Warm water (38°C) normally perfused the chamber. When a water-ethylene glycol mixture at -2°C perfused this chamber, a stable temperature of +2°C to +4°C was measured on the dorsal surface of the spinal cord within 3 to 5 minutes. The effectiveness of the block has been demonstrated by electrical stimulation of the cervical spinal cord (Duggan et al 1977). Spinal conduction was restored by perfusing warm water at 38°C.

**Transection of the spinal cord:** In experiments in
which spinal events alone were being studied, the spinal cord was transected at the lower thoracic level after infiltration with 1 % xylocaine solution.

2.1.3. Brain stem

In order to approach nuclei of the midbrain, the cat's head was fixed in a stereotaxic head frame. The bone of the skull was partially removed within the range of Horsley-Clarke co-ordinates AP +2.0 to AP - 4.0. For stimulating nuclei of the medulla, the head frame was rotated so that the animal's head was ventroflexed, so enabling caudal nuclei to be reached without removing part of the cerebellum. The dorsal surface of the brainstem was exposed by removal of the caudal cranium. After opening the dura, the surface of the brain was covered with either an Agar gel (Agar 4% in 165 mM NaCl) or cotton wool soaked in Ringer solution.

2.2. Antibody Microprobes (Chapter 3)

The antibody microprobe was developed by Duggan and Hendry (1986) in an attempt to produce a detection device which could be introduced to the sites of peptide release in the absence of significant trauma and which could define sites of release with good spatial precision. This method is a modified solid-phase autoradiographic immunoassay. It was felt that the best way to do this was by attaching a binding protein for a ligand to the outside of the fine glass micropipettes normally used by electrophysiologists for
extracellular recording. The most suitable and readily available binding proteins are clearly specific antibodies to the peptides under investigation. Should an antibody bind a released compound then the detection of this combination can be done in several ways. The inhibition of binding of the $^{125}$I-labelled ligand was chosen as the simplest means of detection. In vitro, this can be measured in a gamma counter but for probes introduced into the nervous system, autoradiography is the only means of showing localized zones of inhibition of $^{125}$I ligand binding which would correspond to localized sites of release in vivo. It should prove possible, however, to use the methods of immunocytochemistry to detect bound ligand provided the second antibody recognizes a site on the ligand which differs from that recognized by the antibody bound to the micropipette. The preparation and use of antibody microprobes for the studies reported in this thesis is described below.

2.2.1. **Preparation of the Antibody-coated Microprobes**

Antibodies to SP were bound to glass by a method used for enzyme immobilization (Messing 1975).

A. **Attachment of an organic group to the glass surface:**

The particular technique was first to attach amine groups to external surface of the Pyrex glass micropipettes. Glass tubing (3 mm outer diameter) was drawn out in an electrode puller. The taper was very gradual. Thus, 3 mm from the tip of each pipette the diameter was only 50-60 µm. After sealing of the ends by heating, glass micropipettes were immersed in
a 10% solution of γ-aminopropyltriethoxysilane in toluene for 24 hours. This produced a coating of a siloxane polymer containing free amino groups.

B, Coupling protein A to the glass: rather than couple an antibody directly, protein A was firstly attached to the silanated glass. This protein is derived from staphylococci and preferentially binds antibodies from the complex mixture of proteins present in an antiserum. The constant region (Fc) of antibodies is bound by protein A and thus antibodies can still freely bind ligands. After silanation of glass micropipettes they were immersed in a 2.5% solution of glutaraldehyde for 30 minutes (Fig.1). The coupling of protein A (Sigma) was performed using a 10% solution contained within 5 µl capillaries. Incubation was for 24 hours at 6°C. Following incubation the micropipettes were immersed in 2.5% solution of sodium borohydride to stabilize Schiff bases formed by glutaraldehyde coupling.

C, Coating antibody: Antibodies were finally bound to protein A by allowing 24 h incubation at 6°C. Again this was performed in 5 µl capillaries using a 1 in 20 or 400 dilution of the serum. The anti-serum to SP for the results of the present experiments was obtained from UCB Bioproducts (Brussels). Information supplied by the manufacturer indicates that this antiserum has negligible cross-reactions with somatostatin, Leu- and Met-enkephalin, neurophysins I and II, neurotensin, oxytocin, bombesin, corticotropin-
Fig. 1. Attachment of an organic group and coupling protein A on to the glass surface.
releasing factor, gastrin$_{1-7}$ and cholecystokinin$_{1-8}$.

2.2.2. Detection of SP

A, **IN VITRO**

For in vitro testing the antibody-coated microprobes were immersed in 5µl capillaries containing solutions of synthetic SP (Sigma) with concentrations ranging from $10^{-5}$ to $10^{-11}$M. The incubation time was restricted to 30 minutes (at 23°C) as it was considered that longer incubation times were inappropriate if the method was to be useful physiologically. After incubation, the microprobes were washed for 20 minutes in a phosphate buffered saline (PBS) containing 0.1% Tween. The detection of bound SP involved incubating the antibody microprobes with 1500 - 2000 cpm Bolton-Hunter [$^{125}$I]SP (Amersham) in bovine serum albumin (5mg/ml) to minimize non-specific binding. After incubation for 24-36 h, probes were washed and the bound radioactivity was measured in a gamma counter. Finally, the ends of the antibody microprobes were broken off (10 - 15 mm length) and placed on X-ray (Kodak XRP5) film for autoradiography.

B, **IN VIVO** measurement of release of SP immunoreactivity

Two probes were inserted concomitantly into the spinal cord to depths of 1.5 to 3 mm and remained in place for 5 to 30 minutes under the following conditions: (a) in the absence of any nerve stimulation. (b) when stimulating large myelinated afferent fibers of the ipsilateral tibial nerve with a strength not greater than 1.5 X threshold, and (c)
when using a stimulus strength (100 X threshold) adequate to excite unmyelinated primary afferent fibers of the ipsilateral tibial nerve. All of these procedures were done either in the absence of any protease inhibitors or following the microinjection of 0.5 µl of a solution of mixed protease inhibitors (dissolved in 165 mM NaCl) in the region of the substantia gelatinosa of the dorsal horn to prevent degradation of released substance P. The mixture consisted of leupeptin 10⁻⁶M, aprotonin 10⁻⁶M antipain 10⁻⁶M and pepstatin 10⁻⁶M. Accurate localization of microprobe tips was obtained by filling them with a solution of Pontamine sky blue in Na acetate and ejecting the dye electrophoretically. After removal from the spinal cord, antibody microprobes were washed and incubated in phosphate-buffered saline containing 1500-2000 dpm Bolton-Hunter [¹²⁵I] SP (Amersham) and bovine serum albumin (5 mg/ml) for 24 hours. They were then washed and the tips broken off and placed on X-ray film (Kodak XRP 5) for 24 to 72 hours. In vitro assays were performed in parallel with all in vivo experiments.

C. Analysis of images

The conical nature of the probes produced autoradiographic images showing increasing scattering with increasing distance from the tip. This decrease in resolution was a limitation in assessing the results of in vivo experiments which produced probes with localized zones of inhibition of binding at various sites along their length. This type of image makes accurate quantitation difficult.
Thus, a simple qualitative estimate of the extent of inhibition of $[^{125}\text{I}]$SP binding was chosen. A zone of inhibition of binding of length approximately 100 μm or greater could easily be distinguished within 4 mm of the tips of the microprobes. For in vivo estimates, "O" refers to no inhibition of binding. The estimate "+" refers to submaximal inhibition of binding and "++" to a complete inhibition. Parallel assays were always performed in vitro, and the "++" estimate was approximately equivalent to $10^{-7}$M SP and the "O" estimate to $10^{-9}$M SP.

2.2.3. IN VIVO Preparation

A, Animal preparation

The general procedures have been described in section A of this chapter. Both the left and right tibial nerves were prepared for electrical stimulation. The lumbar spinal cord was exposed, transected at the thoracolumbar junction and covered with an agar gel which was removed at sites of microprobe penetration. Small openings were made in the spinal cord pia for the entry of microprobes and the dorsal surface of the cord was continually irrigated with warm Ringer's solution (containing NaH$_2$PO$_4$, 2H$_2$O, MgCl$_2$, 6H$_2$O, CaCl$_2$, NaCl, KCl, PH 7.4) at 37°C.

B, Stimulation of peripheral afferent fibres

(a) Electrical stimulation of the tibial nerve: A continuous train of 0.3 ms pulses at 10-31 Hz was used commonly. Occasionally a group of 3 pulses (310 Hz) repeated
at 2-10 Hz was employed. Nerve thresholds were measured with a ball electrode placed on the appropriate dorsal roots when the tibial nerve was stimulated. Intensities of 100 to 250 X threshold were used to activate unmyelinated primary afferents.

(b) Noxious heat: The hind paw was immersed in a hot water bath (50°C or 52°C) for 10 to 30 minutes. The temperature of the water bath was controlled by a Braun heating pump. When using hot water, the limb was removed after 10 minutes of immersion and allowed to cool in room air (23°C) for 1 to 2 minutes before reimmersion. No peripheral stimulation and immersion of the hind paw in warm water (37°C) were used as controls.

C, Extracellular recording from microprobes

The tips of antibody microprobes were broken back to 5-10µm diameter and the probes were filled with a 2% solution of pontamine sky blue in 1.2 M sodium acetate for recording extracellularly from neurones. Microprobes were inserted into the spinal cord to depths of 1.5-3 mm through the area which gave the maximal surface potentials, measured with a surface ball electrode, when the ipsilateral tibial nerve was stimulated. Recordings from microprobes during penetration of the spinal cord were also used to ensure that neurones in the vicinity were excited by peripheral stimuli. When a hind paw was immersed in hot water, the increase in firing of neurones produced by this stimulus returned to pre-immersion levels with each period of cooling, indicating that any
possible tissue damage was insufficient to produce sustained firing of nociceptors.

2.3. Recording of electrical activity in the spinal cord

2.3.1. Spinal reflexes (Chapter 4)

Fig. 2 illustrates the arrangement of stimulating and recording electrodes used. The BST, ML-G, sural and tibial nerves were stimulated electrically by means of bipolar platinum wires. Three ventral roots (L₆, L₇ and S₁) were mounted on electrode pairs for differential recording of monophasic reflexes.

For the determination of nerve thresholds (T) to electrical stimulation, and the monitoring of incoming volleys during the recording of reflexes, a ball electrode was placed on an appropriate dorsal rootlet close to its junction with the spinal cord. The stimuli eliciting reflexes have been expressed as multiples of these thresholds.

Following amplification (time constant 300 ms), reflexes were recorded as the averaged responses to 16 stimuli (at 1 Hz) using a signal averager. These averages were recorded on a pen recorder and the areas calculated. Monosynaptic reflexes to stimulation of the BST and ML-G nerves were stabilized by using two closely timed nerve stimuli, the first being subthreshold for a reflex. Reflexes to stimulation of C fibers of the tibial nerve were rarely recorded as large amplitude potentials resulting from summed
action potentials, and usually consisted of relatively widely dispersed individual action potentials. In such cases a window discriminator was used to include all action potentials above a selected baseline and an electronic counter was appropriately gated to count only spikes which, by their latency and the nerve stimulus necessary to elicit them, were produced by impulses in C primary afferent fibers.

2.3.2. Ascending volleys (Chapter 4)

As shown in Fig. 2, the region of the dorsal spinocerebellar tract (DSCT) was chosen for recordings of monosynaptically relayed ascending impulses from group I muscle afferent fibers (Lundberg 1964), when the ML-G nerves were stimulated. In some experiments, the spinal cord was divided at a high cervical level and a pair of ball electrodes placed on the left dorsolateral fasciculus (DLF) for differential recording of ascending volleys, or the spinal cord was transected at the mid-thoracic region and DLF volleys recorded just caudal to the site of section. At high cervical levels there probably are few or no branches of primary afferent fibers from hind limb group I muscle afferents present in the dorsal columns (Brown 1973) and hence recordings over the DLF are unlikely to contain non-synaptically relayed impulses in primary afferent fibers. At the mid-thoracic level this is more likely, so in two experiments, the dorsal columns were divided just caudal to the recording site and in the third it was shown that the potential recorded over the DLF was not recorded by ball
Fig. 2. A diagram of the stimulating and recording techniques. Note that spinal reflexes and volleys to large diameter muscle and cutaneous afferents were recorded as compound action potentials and averaged volleys to C fibre stimulation were recorded as individual spikes using short amplifier time constants and counted by gating an electronic counter.
electrodes placed on the mid-thoracic ipsilateral dorsal column region.

Ascending volleys from impulses in unmyelinated primary afferents were recorded with ball electrodes on the left anterolateral fasciculus (ALF) of the spinal cord at either high cervical, mid-thoracic or upper lumbar level according to the particular experiment (Fig. 2). This region was chosen since it is known that ascending volleys from stimulation of peripheral C fibers can be recorded from this quadrant (Willis & Coggeshall 1978). The ALF recording were obtained from a site one to two segments caudal to the site of cord section. Thus it is highly likely that only ascending impulses were recorded.

The right tibial nerve was stimulated with a stimulus strength greater than 100 T. Ascending impulses in the ALF were recorded with a short amplifier time constant (0.001 or 0.003 s), to reduce the amplitude of compound action potentials, and observed as individual spikes. As with reflexes, a window discriminator was used to select multi-unit recordings. A gated electronic counter was used to count those action potentials which, by their latency and the stimulus strength needed to elicit them, were produced by impulses in unmyelinated afferent fibers of the contralateral tibial nerve. Ascending volleys from electrical stimulation of large myelinated afferent fibers of the contralateral sural nerve (up to 6 T) were also recorded with ball electrodes on the ALF. These volleys were recorded as compound action potentials (with 0.03 to 0.1 s amplifier time
constants) and were averaged. During recording, tissue fluids in the region adjacent to the recording electrodes were continuously or intermittently sucked away.

2.3.3. Extracellular recording (Chapter 3 to 5)

The firing of single neurones in laminae I and III-V of segments L₆ and L₇ were recorded extracellularly with single glass micropipettes or through the centre barrels (filled with 4 M sodium chloride) of 7-barrel micropipettes. Neurones were assigned to the various Rexed laminae on the basis of depth from the dorsal surface of the cord, with electrode tracks being vertical and approximately mid-way between the midline and the dorso-lateral sulcus. In some experiments, in order to record the firing of neurones of laminae IV-V while ejecting drugs in the substantia gelatinosa (SG), two independent micropipette assemblies were used (Duggan, Hall & Headley 1977). The tip of a 7-barrel micropipette was first inserted in the SG using the amino acid-induced firing of neurones of lamina I as guide. A single micropipette was then introduced into the spinal cord at 18° to the vertical such that its tip would be placed 300 µm below the tip of the 7-barrel micropipette when the tips of both were in the same vertical line (Fig.3).

Action potentials were counted with a window discriminator and ratemeter, and cell firing rates were continuously displayed on a pen recorder. In addition, when noxious heat was applied, the total number of action potentials evoked by a period of noxious heat was determined with a microprocessor
Fig. 3. A diagrammatic representation of stimulating and recordings techniques used (A). In B, note that there is the difference between onset times of homoafferent nerve conditioning stimulus (HMS) and heteroafferent nerve conditioning stimulus (HTS) (See text, page 17).
controlled integrator which performed the appropriate baseline (spontaneous firing) substraction. Analogues of these counts were continuously plotted. When neurones were excited by electrical stimulation of nerve, the number of action potentials per stimulus evoked by impulses in unmyelinated primary afferent fibers was counted by appropriately gating an electronic counter. An analogue of each C response was displayed on a pen recorder and the mean (and standard error of the mean) of 16 or 25 responses was calculated during the compilation of a peristimulus histogram of cell firing.

2.3.4. **Intracellular recording** (Chapter 4)

A, **Single micropipette**

Intracellular recordings were obtained with 1.2 M potassium citrate or 2 M KCl-filled glass micropipettes of 10-30 megohm resistance. Estimates of motoneurone membrane conductance were made by the passage of current pulses using the active bridge of a WPI model M701 microprobe unit. Membrane potential was continuously displayed on a pen recorder. Depolarizing membrane potential changes evoked by 16 successive nerve stimuli were summed with a signal averager and the averages plotted on a pen recorder. Samples of spontaneously occurring changes in membrane potential were photographed on moving film using a Grass camera.

B, **Parallel micropipette assemblies**

Electrodes were prepared by cementing a 5- or 7-barrel
assembly (tip diameter 6-8 µm) to a single 1.2 M potassium citrate-filled microelectrode using the technique of Curtis (1968). The adhesive (Loktite I.S. 496) was applied from a fine drawn out polythene piece under microscopic control. The single pipette projected 60-100 µm beyond the tip of the multibarrel assembly for intracellular recording.

Motoneurone membrane potentials were displayed continuously on a pen recorder together with the currents used to eject compounds microelectrophoretically. Depolarizing and hyperpolarizing postsynaptic potentials produced by nerve stimulation were recorded by averaging 16 responses and then plotted on a pen recorder.

The data presented in this thesis is restricted to motoneurones with resting membrane potentials greater than -50 mV and shown to be depolarized by microelectrophoretic D,L-sodium homocysteate (DLH) (40 nA). The latter is important since failure of a compound to affect membrane potentials in experiments of this type can result from tissue debris preventing free diffusion of ejected compounds, partial separation of the tip of the drug administering pipette from the recording pipette as well as inactivity by the relevant compound.

2.4. **Stimulation** (Chapter 4 to 5)

2.4.1. **Peripheral stimulation**

The arrangement of electrical and "natural" stimulation is shown in Fig. 3.
A, Test stimulation

(a) Peripheral nerve: electrical stimulation (a pulse of 0.3 ms) of the tibial nerve, repeated at 0.2 - 0.6 Hz, was suprathreshold for unmyelinated (C) afferent fibers.

(b) Noxious heat: noxious heating of the glabrous skin of the left plantar region was produced by an electronically controlled lamp to temperatures in the range 45° - 55°C. A thermo-couple on the skin measured surface temperature and provided feedback control of the heating lamp (Duggan, Hall & Headley 1977).

(c) Non-noxious touch: deflection of hairs adjacent to the heated area by a moving air jet was used as an innocuous stimulus.

B, Conditioning stimulation

(a) Homoafferent nerve conditioning stimulation: the conditioning stimulus (0.2ms pulse, 50-100Hz, for 10-20 sec) ceased just prior to the nociceptive response, as shown in Fig.3B.

(b) Heteroafferent nerve conditioning stimulation: the conditioning stimulus was applied throughout the evoked response (Fig.3B).

2.4.2. Brain stimulation (Chapter 5)

A, Stimulation of the periaqueductal grey matter (PAG)

Electrical stimulation in the PAG was performed with a pair of steel needles (0.15 mm diameter, insulated to 0.5 mm of the tip) 3 mm apart and aligned in the transverse plane.
The electrodes were introduced in the Horsley-Clarke vertical plane across the midline and positions were determined by standard stereotactic techniques using the atlas of Berman (1968). Stimulation sites were confirmed histologically by iron deposition from the electrode tips. Bipolar stimulation consisted of a tetanus of 0.2 ms pulses at 310 Hz for 100 ms, repeated at the same frequency as the peripheral nerve stimulus activating the dorsal horn neurone. Thus during stimulation of brainstem structures, each peripheral stimulus and counting gate was preceded by a tetanus, which was timed to produce significant but submaximal inhibition of the gated response. The last pulse of the train occurred within 10 ms before the start of the counting gate. The stimulus current (range 20 -200 µA) was monitored continuously.

B. Stimulation of the dorsolateral pons (LC and NKF)

Electrical stimulation of the dorsolateral pons was performed with a pair of steel electrodes (0.15 mm diameter, insulated to 0.5 mm of the tip) 2.5 mm apart and aligned in the transverse plane. The electrodes were inserted into the regions of the locus coeruleus (LC) and the nucleus Kolliker-Fuse (NKF) at an angle of 45° to the Horsley-Clark horizontal plane and positioned at the stereotaxic coordinates P 2.0--4.0, L 2.0 -3.0, V 2.0 - 2.5 using the atlas of Berman (1968). Since the LC surrounds the mesencephalic nucleus of the trigeminal nerve, the jaw opening reflex produced by electrical stimulation of this area was used as an aid to placement of the electrodes (Sasa & Takaori 1973). As with
the PAG, stimulation sites were confirmed by iron deposition from the electrode tips.

Monopolar stimulation (a tetanus of 0.3 ms pulses at 300 Hz for 20-100 ms) was delivered through one electrode of each pair of stimulating electrodes. A ground electrode was attached to muscle surrounding the cranium. When attempting to inhibit the C responses of dorsal horn neurones, to avoid false counting of stimulus pulses, the last pulse of each tetanus to the LC occurred immediately prior to the onset of the gate used for counting C responses. With excitation of neurones by noxious heating of the skin, a continuous tetanus (50 Hz) was used to stimulate in the region of the LC or the NKF throughout the period of skin heating. Stimulation current (50-300 µA) was monitored continuously.

2.5. Administration of drugs

2.5.1. Microelectrophoresis

This method is appropriate for the study of synaptic pharmacology in the CNS and consists of movement of charged molecules of drugs produced by passage of an electric current from glass micropipettes to the extracellular environment of a neurone. The procedure used has been described by Curtis (1964).

Seven barrel micropipettes were drawn in a vertical microelectrode puller. Usually, an overall tip diameter of about 1 µm was initially produced and then broken back to an overall diameter of 5 - 7 µm by striking the tips with a fine
glass rod. This diameters of the tips used represents a compromise between the need to reduce spontaneous drug diffusion from large openings, and the poor current-passing characteristics of barrels with smaller tips.

The solutions used were firstly centrifuged, and then transferred into individual barrels of the micropipette. After further centrifugation, the adequacy of filling of barrels was inspected by microscopy and the electrical resistance of each was measured.

The centre barrel of each micropipette was filled with 4 M sodium chloride. The aqueous solutions of drugs contained within the outer barrels of the micropipettes are listed in Table I.

To determine the resistance of each barrel of a micropipette in the spinal cord, the current required to maintain 0.5 V potential difference across the solution was measured. The polarity of this voltage was to retain the active ion within the micropipette and was opposite to that applied for ejecting the same ion. If a seven barrel micropipette is located extracellularly very close to a neuronal membrane, the passage of current from a barrel to ground may be sufficient to result in a change of neuronal excitability. Thus, to minimize this problem, one barrel of each micropipette was filled with 100 mM NaCl for current balancing.

2.5.2. Intravenous injection

Solutions for intravenous administration were as
follows.

Naloxone HCl 0.1 mg/ml,
N-(3-furylmethyl)-α-nor-metazocine methanesulphonate (FMN) 1.0 mg/ml,
Idazoxan HCl 1.0 mg/ml,
Clonidine HCl 0.2 mg/ml,

All of these compounds were dissolved in 165 mM NaCl, and were injected into the left radial vein.

2.5.3. Microinjection

In some experiments, the excitant amino acid Na-L-glutamate (50 or 500 mM) was injected in the pons in volumes of 0.1-0.2 µl with an injection needle (30 gauge, 0.35 mm o.d. attached to a micrometer-driven syringe) which was electrically insulated except for the tip exposure (0.5 mm). Thus, it was possible to compare the effects of alternate chemical and electrical stimulation at the one site as an aid to distinguish between excitation of fibers of passage and cell bodies.

2.5.4. Topical application

Idazoxan at a concentration of 10 to 50 mM was gently pipetted onto the surface of the spinal cord. After each test, the drug was sucked away and the cord was washed with Ringer's solution.

2.6. Localization of electrode tips and lesions of the brain
2.6.1. Localization of tips

A, Micropipette

The micropipette was filled with a 2% solution of pontamine sky blue in 1.2 sodium acetate. The dye was ejected as an anion at 5 µA for 5 - 10 minutes at the termination of recording (Stone 1985).

B, Steel electrode

Current was passed between the stimulating steel electrode (made the cathode) and ground using 10 to 20 µA for 60 seconds. The Prussian blue test was used to determine the localization of tips.

C, Histology

Neuronal tissue containing the dye deposits was fixed in 10% formal saline for three days. Fifty µm transverse frozen sections were cut and examined by microscopy.

2.6.2. Electrocoagulation

An array of 4 needles separated by 1.0 - 1.5 mm and aligned in the longitudinal plane was used to coagulate medullary regions. Using the atlas of Berman (1968), these electrodes were positioned stereotactically after obtaining a manipulator midline reading by aligning the array directly over the dorsal median fissure of the exposed brainstem, which was then covered with a saline-agar gel. High frequency alternating current (20 - 50 mA at 500 KHz for 15 s) between adjacent electrode pairs was used to coagulate brainstem regions. The location and extent of destruction were subsequently determined by gross examination of the fixed brainstem and of 15 µm transverse paraffin sections.
CHAPTER 3  RELEASE OF SUBSTANCE P FROM PRIMARY AFFERENT TERMINALS IN THE SPINAL CORD

3.1. INTRODUCTION

"The discovery of substance P was unexpected but not wholly fortuitous"--U.S.Von Euler (1977)

The present work used the newly developed antibody microprobe (Duggan and Hendry 1986) to define sites at which afferent impulses produced a release of substance P (SP) in the spinal cord. As a background to these experiments, it is appropriate to discuss the evidence which suggests that SP may be a transmitter released from the central terminals of primary afferents.

Fifty years ago, Von Euler and Gaddum discovered the presence of a powerful vasodilator substance, the action of which was not blocked by atropine, in extracts of brain and intestine and which they termed substance P (SP) (Von Euler & Gaddum 1931, Von Euler 1977). Twenty years later, the levels of SP were found to be higher in the dorsal roots than in the ventral roots (Amin, Crawford & Gaddum 1954, Lembeck 1953, Pernow 1953). Based mainly on this finding, Lembeck (1953) proposed that SP might be a transmitter released by the central terminals of primary sensory neurones. In 1971, Leeman and her colleagues finally determined the structure of SP as an undecapeptide (Fig.4) and synthesized it (Chang, Leeman & Niall 1971, Leeman & Carraway 1977). Nowadays, SP
Fig. 4 Amino acid sequence of substance P (From Skrabanek & Powell. 1977).
has become the best documented of an increasing group of neuropeptides. An average of one paper on SP is published every day (Pernow 1983). As a result, its actions and possible physiological functions have been widely studied. It is known that SP is found in several organ systems, including the central nervous system (CNS), gastrointestinal, cardiovascular and salivary glands systems (Pernow 1983). This review will focus only on the possible involvement of SP in sensory transmission, especially nociceptive transmission in the mammalian spinal cord.

3.1.1. The Tachykinin Family and Substance P Receptors

SP is a member of a family of natural occurring peptides known as the tachykinins, which have similar biological activities and share a common C-terminal sequence, namely, \(-\text{Phe-X-Gly-Leu-Met-NH}_2\), where X is a hydrophobic or aromatic residue.

Substance P: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$
Phyalsaemin: pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH$_2$
Eledoisin: pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH$_2$
Kassinin: Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH$_2$
Neurokinin A: His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH$_2$
Neurokinin B: Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH$_2$

Based on an analysis of the rank order of potency for the
effects of tachykinins on a variety of tissues, Erspamer's group first proposed the presence of more than one type of tachykinin receptor in vertebrates (Erspamer et al 1980, Erspamer 1981). They found that kassinin and eledoisin were more potent than SP or physalaemin in stimulating certain types of smooth muscle, whereas the latter peptides were more potent than kassinin and eledoisin in their effects on blood pressure or salivation. From the responses of a wide range of pharmacological test systems, Iversen's group proposed the presence of two SP receptors sub-types (Iversen et al 1982a,b, Lee et al 1982):

1. termed the SP-P type, has the rank order of potency:
   Physalaemin > SP > Eledoisin > Kassinin

2. termed the SP-E type, has the rank order of potency:
   Eledoisin = Kassinin > SP = Physalaemin

Bioassay potencies of these tachykinins are essentially equipotent at the SP-P system, whereas the SP-E system is more sensitive to eledoisin and kassinin than to SP and physalaemin. Thus, SP generally is accepted as being the endogenous ligand for the mammalian SP-P receptor.

Although the classification of SP receptor sub-types seems to be accepted, it remains tentative. In particular, since most of the evidence in support of the existence of SP receptor sub-types has been obtained from studies on peripheral tissues, more information is required to determine whether this classification may be entirely suitable for SP receptors in the CNS. As mentioned above, SP, physalaemin and kassinin are almost equipotent on SP-P receptors in
peripheral bioassay (Lee et al 1982). By contrast, the latter two compounds have a low binding affinity to brain SP-P receptors (Iversen et al 1982b, Torrens et al 1983, Quirin et al 1983a). These observations suggest the existence of a difference between peripheral and central SP receptor subtypes. However, in competition binding experiments using $[^3H]SP$, $[^{125}I]SP$ and $[^{125}I]$ physalaemin, both SP and physalaemin were very potent in displacing all three ligands in the rat brain. By contrast, eledoisin was much less potent than SP and physalaemin in displacing these radioactive ligands (Quirin et al 1983b). A similar result was obtained from a study on inhibition of $[^{125}I]SP$ binding in the rat spinal cord. The order of potency was SP > physalaemin > eledoisin (Charlton & Helke 1985). In addition, in behavioral studies (Moochhala & Sawynok 1984, Cridland & Henry 1986, Games & Saria 1986), it has been found that intrathecal application of tachykinins decreased reaction time in the rat tail flick test. Analysis of the dose-response curves indicated that the rank order of potency of the fitted curves for these peptides was physalaemin > SP > eledoisin. All results are quite similar to those from peripheral tissue cited above, suggesting that the SP-P receptor occurs in the CNS.

Until recently, SP was the only member of the tachykinin family found in the vertebrate CNS. Three years ago, two novel tachykinins, termed neurokinin A (also known as neurokinin α, neuromedin L or substance K) and neurokinin B (also known as neurokinin β or neuromedin K) were isolated
from porcine spinal cord and chemically identified (Kangawa et al 1983, Kimura et al 1983, Minamino et al 1984 1983). In contrast to SP, both neurokinin A and neurokinin B were very weak competitors for $[^{125}\text{I}]$SP binding sites (Quirin & Pilapil 1984, Kimura et al 1984). In pharmacological studies (Kimura et al 1984), both neurokinin A and neurokinin B exerted similar or a little weaker actions on the guinea-pig ileum than SP (where SP-P receptors predominate) but much more potent actions on the rat vas deferens (where SP-E receptors predominate) (Kimura et al 1984). In the rat tail flick test, intrathecal administration of neurokinin A was considerably less potent than SP, physalaemin and eleedoisin in prolonging latencies (Cridlund & Henry 1986). These results indicate that neurokinin A and neurokinin B may act preferentially on the SP-E receptor. As a consequence, these two neuropeptides have been characterized as SP-E receptor ligands (Kimura et al 1984, Hunter & Maggio 1984, Nawa et al 1984). Recently a third sub-type of tachykinin receptor, SP-K, has been described in gastro-intestinal and urinary bladder smooth muscle membrane (Buck et al 1984) and in the substantia nigra (Quirion & Dam 1985), for which the potency rank order is neurokinin A > kassinin > eleedoisin > neurokinin B > SP > physalaemin. Thus, neurokinin A may be an endogenous ligand at SP-K receptors.

Using autoradiographic techniques, the localization of SP receptors has been studied in the spinal cord of the rat (Quirion et al 1983a, Maurin et al 1984, Charlton & Helke 1985, Ninkovic et al 1985) and the ox (Mantyh & Hunt 1985).
The lumbosacral spinal cord bound 3 times more than cervical and thoracic segments. The highest concentration of SP receptors is located in lamina X, while moderate to high concentrations are located in laminae I,II and V-IX. A very low concentration of SP receptors is present in laminae III-IV. This distribution approximately parallels the sites of termination of nociceptive primary afferents.

3.1.2. Substance P as a transmitter candidate in the spinal cord

The present concepts of chemical transmission are derived largely from the study of the neuromuscular junction. In a discussion of SP as a putative transmitter in the CNS, we still follow these basic criteria which are widely accepted: the substance should be present in the presynaptic neurones; the substance should be released from the presynaptic neurones; and the substance should produce physiological responses identical with those produced by stimulation of presynaptic neurones.

A. Distribution of Substance P in the Spinal Cord

As many as 30 groups of SP immunoreactive (SP-IR) cell bodies are found in brain (Ljungdahl, Hokfelt & Nilsson 1978). In the spinal cord, SP-containing fibres are widely distributed whereas SP-containing cell bodies are located only in the dorsal horn (Hokfelt et al 1975, 1976,1977a) and in the dorsal root ganglia (Hokfelt et al 1975, Takahachi & Otsuka 1975).
In the 1950s, using bioassay techniques, several groups found that the levels of SP in dorsal roots were greater than those in ventral roots (Lembeck 1953, Pernow 1953, Amin, Crawford & Gaddum 1953). From these results, SP was proposed as a sensory transmitter by Lembeck (1953). Subsequently, high concentrations of SP in the dorsal part of the spinal dorsal horn of the cat were measured by the group of Otsuka using bioassay (Takahachi & Otsuka 1975). In addition, intense SP immunofluorescent staining was observed in about 20% of DRG cells in the cat (Hokfelt et al 1975). More recent studies using radioimmunoassay have confirmed the difference in SP levels between dorsal roots and ventral roots, and also the existence of a gradient in SP content along the central processes of primary afferents. The level of SP in dorsal roots was 9-27 times higher than that in ventral roots, and the levels of SP in rat spinal ganglia, dorsal roots and the lumbar dorsal horn were 43.7, 88.4 and 557.1 pmol per g wet tissue, respectively (Ogawa, Kanazawa & Kimura 1985).

The concentration of SP immunoreactivity in the dorsal horn of the rat is 4-10 times higher than that in the ventral horn (Brownstein et al 1976, Jessell et al 1979, Ogawa, Kanazawa & Kimura 1985). With the immunohistochemical technique, Hokfelt et al (1975,1977b) have demonstrated the detailed distribution of SP-positive fibres. The highest density of SP fibers is found in the superficial dorsal horn (laminae I and II) (Hokfelt et al 1975) where Aδ and C afferent fibres arborize and terminate (Rethely, Light & Perl
1977, Light & Perl 1979, Mori 1986). A lower density of SP-positive fibres occurs in the ventral part of lamina IV, laminae V, VII and X as well as in the ventral horn. A near lack of SP-positive fibres occurs in lamina III and dorsal lamina IV. SP-IR is associated with large dense-core vesicles in terminals located in laminae I and II of the dorsal horn (Bresnahan, Ho & Beatlie 1984, Difiglia, Aronin & Leeman 1982), and SP-positive terminals in most cases form axodendritic and axosomatic synapses with dorsal horn neurones (Barber et al 1979). To examine the origin of these SP-containing fibres, ligation or section of the dorsal roots has been performed. After the operation, the level of SP in the dorsal horn is markedly reduced by 60-70%, suggesting that most originates from primary sensory neurones (Takahashi & Otsuka 1975, Hokfelt et al 1975, Jessell et al 1979, Tessler et al 1981). The distribution of SP in human spinal cord is similar to that just described, with an intense band of SP-containing nerve terminals in laminae I and II of the dorsal horn (Cuello, Polak & Pearse 1976, LaMotte & De Lanerolle 1981).

In addition to SP contained within primary sensory neurones, there is a significant SP-containing descending projection from the brainstem to the spinal cord (Chan-Palay Jonsson & Palay 1978, Hokfelt et al 1978, Bowker, Steinbusch & Coulter 1981, de Lanerolle & La Motte 1982) and SP-containing axons from intrinsic cells of the spinal cord (Hokfelt et al 1975, Barber et al 1979, Kawatani, Erdman & De Groat 1985). Following spinal transection at a mid-thoracic
segment, the content of the SP in the ventral but not the
dorsal horn was obviously reduced (Ogawa, Kanazawa & Kimura
1985, Hokfelt et al 1977b), indicating that SP terminals in
the ventral horn may mainly originate from supraspinal sites.
However, most of SP present within the intermediate and
central canal area (lamina X) of the spinal cord is thought
to arise from intrinsic cell bodies since levels in these
areas are unaltered either by dorsal rhizotomy (Hokfelt et al
1975) or by a combination of dorsal rhizotomy and ipsilateral
transverse hemisection of the spinal cord (Barber et al 1979).

In view of the similarities in the distributions of SP
receptors, SP-containing terminals and primary afferent
fibres conveying nociceptive information, it is not
unreasonable to pursue a possible nociceptive function of SP
in the spinal cord.

B, Release of SP in the Spinal Cord

(a) Studies in vitro

A number of preparations and methods have been used
to study SP release in the spinal cord.

In the isolated newborn rat spinal cord, Otsuka and
Konishi (1976) first demonstrated that electrical stimulation
of the dorsal roots or elevation of the potassium
concentration in the perfusate elicited an increased release
of SP-like immunoreactivity. The stimulus-induced SP release
was eliminated by a low calcium (0.2 mM) and a high magnesium
(7 mM) concentration in the perfusate. This result was
confirmed in a DGR cell culture preparation (Mudge, Leeman &
Recently, the immunoreactive SP release from the isolated rat spinal cord in Krebs solution containing 90mM K\textsuperscript+ and 2.4mM Ca\textsuperscript{++} has been identified as the undecapeptide SP (Akagi, Otsuka & Yanagisawa 1980). A similar depolarizing potassium-induced SP-IR release was also obtained in slices of rat spinal trigeminal nucleus (Jessell & Iversen 1977).

Capsaicin is another agent which releases SP in the spinal cord. This compound caused a calcium-dependant SP release from rat spinal slices (Gamse, Molnar & Lembeck 1979, Bucsics & Lembeck 1981) and the isolated rat spinal cord (Theriault, Otsuka & Jessell 1979), but did not evoke the release of GABA or glycine (Akagi, Otsuka & Yanagisawa 1980). Capsaicin also increased a calcium-dependant SP release in a slice preparation of the spinal trigeminal nucleus in the rat (Helke, Jacobowitz & Thoa 1981). In the chronic capsaicin treated rat there was a marked depletion of substance P immunofluorescence from the dorsal horn of spinal slices (Jessell, Iversen & Cuello 1978). Since the spinal dorsal horn and the spinal trigeminal nucleus are associated with the integration of sensory information, the function of SP in sensory transmission is strengthened. However, due to the limitation of the technique used, in all of these experiments the source of SP release remains unknown.

(b) Studies in vivo

Release of SP-IR from the mammalian spinal cord of the cat in vivo was first studied by Yaksh et al (1980).
Stimulation of the sciatic nerve at an intensity exciting only Aαβ afferents did not significantly increase the spontaneous efflux of SP from the superfused cat spinal cord. Increasing the stimulus intensity to activate both Aδ and C afferents elicited a fivefold increase of SP release. In the same preparation, SP-IR was also released by capsaicin, which is consistent with in vitro results. In another study, stimulation of a bulbospinal pathway by administration of the excitant kainic acid into the ventral medulla evoked the release of SP into a spinal superfusate in the rat (Takano et al 1984).

These results cannot differentiate between a release of SP from primary afferent terminals and that from supraspinal neurones, since the SP-containing terminals of brainstem neurones project to the spinal cord. To examine this problem, capsaicin or 5,6-dihydroxytryptamine (5,6-DHT) was administered intrathecally in the rat (Yaksh et al 1979). Capsaicin was used to deplete SP from sensory afferents. 5,6-DHT was used to destroy serotonin-containing brainstem neurones, and, based on coexistence of 5-hydroxytryptamine (5-HT) and SP in the same neurones of the brainstem raphe system (Hokfelt et al 1980, Gilbert et al 1982), this toxin might be expected to destroy many SP-containing spinal terminals of supraspinal origin. After 7 days of treatment by these neurotoxins, the lumbar spinal cord was removed and the concentration of SP was measured by radioimmunoassay. With capsaicin treated animals, IR-SP content in the lumbar spinal cord was reduced by 55%. In animals treated with 5,6-
DHT, there was a 50% depletion in IR-SP content. Although the result indicated that SP-containing primary afferents and descending fibres probably contribute equally to the concentration of SP in the spinal cord, it did still not determine the origin of SP release when exciting primary afferent fibres. Further, release of SP from superfused rat spinal cord in response to a 10 min pulse of 40 mM KCl was studied in animals pretreated with capsaicin and 5,6-DHT (Jessell 1982). In animals treated seven days earlier with capsaicin, SP release from the spinal cord in response to KCl was 25% of that in rats treated with saline containing 50% dimethylsulphoxide (DMSO) alone. In contrast, the release of SP in rats pretreated with 5,6-DHT was not significantly different from that in control rats. Combined pretreated with both capsaicin and 5,6-DHT, the release of SP was almost completely eliminated. A recent study in the cat also showed that intrathecal 5,6-DHT failed to produce any change in the release of SP from the superfused spinal cord by peripheral stimulation, and that cold block of the cervical spinal cord resulted in a mild increase in the level of SP-LI released from the lower spinal cord by somatic stimulation (Go & Yaksh 1986). These results suggest that SP release originates predominately from sensory afferent fibres.

These studies employed electrical stimulation of peripheral nerve to release substance P, but clearly more physiological stimuli are needed if SP is to be associated with nociception. Recently, Go and Yaksh (1986) found that intense thermal stimulation (55°C) of the skin resulted in a
small increase (about 40%) in the nonstimulated levels of SP-LI in the superfused spinal cord. By contrast, using a push-pull cannula, Kuraishi et al found that noxious cutaneous mechanical, but not thermal, stimuli reproducibly increased the release of immunoreactive SP from the superficial dorsal horn of the rabbit spinal cord (1985a) and that noradrenaline blocked mechanical stimuli-induced SP-IR release (1985b). The cannula used in these experiments was 600 µm in diameter and thus must cause considerable damage in the dorsal horn. Even if the SP released is derived from primary afferent terminals, the push-pull cannula is too coarse an instrument to localize the source of release with any precision. These experiments of Go & Yaksh (1986) and Kuraishi (1985) are in disagreement as to whether noxious heat produces a central release of SP.

C. Depolarizing action of SP on Spinal Neurones

In electrophysiological experiments both in vitro and in vivo, a depolarizing effect of SP on spinal neurones has been consistently demonstrated.

(a) Postsynaptic Action of SP

IN VITRO: Otsuka and his colleagues first found that SP depolarized motoneurones in an isolated frog spinal cord (see references in Otsuka et al 1982). Subsequently, in the isolated rat spinal cord preparation, administration of SP to the perfusing fluid elicited a depolarization accompanied by
spike discharges of motoneurones (Konishi & Otsuka 1974). Comparing the dose-response curves of L-glutamate and SP, the concentrations of SP producing a depolarization were about 200 times (Konishi & Otsuka 1974), even 1000-9000 times (Otsuka & Konishi 1977), less than those of L-glutamate having a similar action. The finding that the depolarizing action of SP still occurred after blockade of synaptic transmission by tetrodotoxin (TTX) (Nicoll 1978, Suzue, Yanaihara & Otsuka 1981) and by lowering calcium in the the perfusate (Konish & Otsuka 1974, Suzue et al 1981) indicated a direct action of SP on motoneurones. Similarly, in the neonatal rat spinal cord slice preparation, bath or microelectrophoretic application of SP produced powerful excitation of almost all dorsal horn neurones recorded extracellularly. When perfused with a Ca²⁺-free, Mg²⁺-high Krebs solution, the excitatory effect of SP was not significantly altered (Otsuka, & Yanagisawa 1980). Intracellular recording from dorsal horn neurones of the rat spinal cord slice demonstrated that SP or repetitive stimulation of a dorsal root elicited a similar slow depolarization in the same neurones which was markedly depressed or abolished by a SP antagonist or capsaicin (Urban & Randic 1984). More recently, Randic et al (1986) further indicated that both poly- and mono-clonal antibodies to SP significantly decreased the amplitude and duration of the slow depolarization generated in dorsal horn neurones by high strength repetitive dorsal root stimulation or exogenous SP. These results also suggest that substance P acts directly at
postsynaptic sites.

**IN VIVO**: Using extracellular recording methods, several groups have found that microelectrophoretic administration of SP excites spinal neurones (Henry 1976, Randic & Miletic 1977, Krnjevic 1977). Using intracellular recording and microelectrophoretic administration of SP extracellularly, a slow membrane depolarization of spinal motoneurones of the cat has been observed (Krnjevic 1977, Zieglgansberger & Tulloch 1979). Limited observations have been made of membrane changes induced in dorsal horn neurones of the cat by microelectrophoretic substance P. Sastry (1979) reported that microelectrophoretic administration of SP (100-450nA) produced a 5-14 mV depolarization of 15 of 18 nociceptive neurones in lamina V of the dorsal horn which had a slow onset, taking 20-40 sec to develop, and persisted for 2-9 min following the termination of the application. Repeated application of SP resulted in a reduction in this effect on the membrane potential. A similar result was obtained by Zieglgansberger & Tulloch (1979). It was found that SP (50-450 nA) caused a relatively slowly increasing depolarization (5-40 mV, reached within 1-3 min) of 6 of 8 dorsal horn neurones.

These results suggest a postsynaptic action of SP, although it has not been possible to apply the further tests outlined above when discussing in vitro results. Support for a postsynaptic action of SP is provided by anatomical studies that SP terminals predominantly make axo-dendric or axo-somatic synapses on dorsal horn neurones (Barber et al 1979).
Microelectrophoretic application of SP into the SG, an area which has a dense plexus of SP containing fibres, depressed the responses to noxious stimulation of deeper dorsal horn neurones with bodies in deeper laminae (Davies & Dray 1980). The authors did not favour this inhibition as resulting from excitation of local inhibitory interneurones since neither DLH nor acetylcholine administering into the SG had any significant effect on neurones depressed by SP. One explanation is that SP acted presynaptically to reduce the amount of transmitter released by impulses in nociceptive afferents.

(b) Effect of SP on Primary Afferent Fibres

To examine directly whether SP exerts a presynaptic action on primary afferent fibres, measurements of thresholds for antidromic activation of primary afferent fibres have been made in vitro (Hental & Fields 1983) and in vivo (Randic et al 1982). Changes in the excitability of nerve terminals are considered to reflect changes in terminal membrane potential and hence in alterations in the amounts of transmitter released (Levy 1980). In the cat spinal cord, SP, applied by pressure microinjection, microelectrophoresis or systemic injection, produced dose-related reversible decreases or increases in the threshold for antidromic activation of both single sural afferent C- and A-fibres (Randic et al 1982). No attempt was made to locate and identify terminal region of these fibres, and the failure to observe consistent effects of SP may merely reflect the
technical difficulties associated with this type of investigation. The changes observed, however, may indicate alterations in activity at axo-axonic synapses on Aδ and C terminals produced by underlying excitatory and depressant actions of SP on spinal dorsal horn interneurones. In the isolated spinal cord of the neonatal rat, Dental & Fields (1982) have reported that bath applied SP only decreases afferent terminal thresholds. The authors considered that this increase in excitability may be accounted for by a model in which SP, GABA and possibly other depolarizing agents are released by interneurones which synapse on afferent terminals.

This possible presynaptic action of substance P is in conflict with anatomical findings. There are very few axo-axonic synapses between dorsal horn neurones and primary afferent terminals in laminae I and II of the spinal cord (Ralston & Ralston 1979). However, ultrastructural studies have found aggregations of large SP-containing vesicles remote from synapses (Barber et al 1979).

Electrophysiologically, the action of the peptide LHRH on neurones of bullfrog paravertebral ganglia is not dependant upon direct contacts between peptide-containing presynaptic terminals and postsynaptic sympathetic neurones (Jan, Jan & Brownfields 1980). Furthermore, GABA, which does synaptically depolarize the terminals of low threshold muscle afferent fibers has a non-synaptic depolarizing effect on DGR neurones (see Curtis & Lodge 1982). It is, therefore, possible that SP could act on primary afferents despite the lack of an
anatomical (synaptic) basis, but the functional significance of such an action would be difficult to establish.

(c) Ionic mechanism of SP-induced depolarization

**IN VITRO**: With intracellular recording from the isolated frog spinal cord, SP-induced depolarization of motoneurones was linked to a small increase in membrane conductance which was considered to result from a sodium conductance increase (Nicoll 1978). A similar result was obtained from sympathetic neurones (Dun & Jiang 1979). In contrast to these observations, it has been reported that depolarization by SP is accompanied by a decrease in the membrane conductance of cultured spinal neurones (Hosli et al 1981, Nowak & MacDonald 1981, Murase, Nedeljkov & Randic 1982). Using a K⁺-sensitive microelectrode, perfusion of a culture of spinal neurones with SP revealed no change of extracellular [K]₀ during the depolarization by SP (Hosli et al 1981). Intracellular ejection of tetraethylammonium, a K⁺ channel blocker, abolished SP-induced deolarization (Nowak & MacDonald 1981). These findings suggest that the depolarizing action of substance P may be due to a decrease of a potassium conductance. Recently, with a microelectrode voltage-clamp technique, slow persistent inward currents were recorded from rat spinal dorsal horn neurones during depolarizing voltage commands before, during, and after the bath application of SP (3 X 10⁻⁷M) in slice preparation (Murase, Ryu & Randic 1986). Substance P augmented a
persistent slow inward Ca\textsuperscript{++}-sensitive current in a dose-depandan
t manner, suggesting that it may be relevant to the SP-induced slow depolarization.

**IN VIVO**: It was found that SP-induced depolarization of cat motoneurones was associated with a decrease in membrane conductance. The reversal potential for this depolarization was negative to the resting potential, suggesting that SP may act by reducing a membrane conductance to K\textsuperscript{+} or Cl\textsuperscript{-} ions with a negative equilibrium potential (Krnjevic 1977). However, a depolarization by SP of cat motoneurones without detectable alterations of membrane resistance, antidromic action potentials or postsynaptic potentials has also been observed (Ziegglansberger & Tulloch 1979). Additionaly, during the SP-induced depolarization, the membrane resistance, measured by brief hyperpolarizing current pulses, was not significantly altered (Sastry 1979).

To date analyses of the possible synaptic actions of SP are still confusing. The conflicting results in vitro and in vivo may derive from technical problems and the use of different preparations.

Taking into consideration all of the evidence outlined above on distribution, release and actions, SP has fulfilled most of the major criteria for qualifying as a transmitter of some primary afferents terminating in the spinal cord. Nevertheless, the slow time-course of SP actions appears to
be inconsistent with a fast postsynaptic action, for example, by the transmitter released by Ia terminals on motoneurones (Eccles 1964). Such differences are known with other compounds e.g. the muscarinic action of acetylcholine (McGeer, Eccles & McGeer 1978). As with SP, a long latency slow EPSP recorded in sympathetic ganglion cell of the bullfrog is mediated by an LHRH-like peptide. The histology of this site suggests that the peptide must diffuse microns before reaching a ganglionic neurone (Jan & Jan 1982). In support of this hypothesis that chemical interaction between neurones in the CNS can occurs in the absence of well defined synaptic complexes, more recently, Zhu et al (1986) demonstrated exocytotic release from large dense cored vesicles at structurally nonspecialized areas in rat trigeminal subnucleus caudalis. In rats the marginal layer and the SG of the medulla contain the central terminals of primary afferent fibers from the infraorbital nerve that supply the skin and whiskers (vibrissae). After a skin lesion in the vibrissa area, an increase in large vesicle exocytosis at structurally non-synaptic sites and a decrease in level of SP-LI were concomitantly produced by lesions of the skin in whiskers (vibrissae) area, which suggests a release of SP from sites other than the active synaptic zone. It is conceivable that its long lasting action possibly derives from diffusion of SP released from non-synaptic zone.

3.1.3. Substance P and the central terminals of peripheral nociceptors
Evidence that substance P is possibly involved in the transmission of information from the spinal central terminals of nociceptors has come from a variety of experimental techniques. The results from some of these are summarized below.

A. Anatomical evidence

As mentioned above, SP is located in approximately 20% of small (B type) DRG cells (Hokfelt 1983) and in a dense plexus of terminals in spinal lamina I and the outer part of the SG with smaller amounts in lamina V. Importantly, SP is found in the central terminals of glomerular structures of the superficial dorsal horn (Barber 1979, LaMotte & de Lanerolle 1983). The central terminal of each such glomerulus is considered to be a C or Aδ primary afferent fibre (Gobel 1979), many of which are the terminals of nociceptors. This anatomical evidence gives a structural basis for a role for SP in transmission of information from nociceptors to spinal neurones.

B. Behavioral studies

In behavioral experiments, intrathecal injection of SP produces changes consistent with hyperalgesia. A reduction in reaction latency was obtained in the rat hot plate test (Hayes & Tyers 1979, Akerman, Rosell & Folkers 1982), the tail withdrawal test (Lembeck, Folkers & Donnerer 1981), the biting and scratching test (Hyder & Wilcox 1981, Piercey et al 1986, Gamse & Saria 1986) and the tail flick
test (Yasphal, Wright & Henry 1982, Sawynok et al 1984, Sawynok & Robertson 1985, Gamse & Saria 1986). Absence of SP might be expected to produce hypalgesia. Supporting this is the remarkable report of Pearson et al (1982) in which it was shown that there was an almost complete absence of SP-IR terminals from the SG in all patients with familial dysautonomia, a neurological disorder, associated with a severe reduction of in pain sensitivity. In contrast, in patients with chronic arachnoiditis, a condition associated with chronic low back and leg pain, the levels of SP in the lumbar CSF were much higher than those of normal subjects. Morphine concomitantly reduced both the pain and the high SP levels (Hosobuchi 1981). All of these results suggest that there is an association between SP and nociception. But, a contrary result, that intrathecal SP increased reaction latency in the rat tail flick test, has been reported (Dio & Jurna 1981).

If SP is involved in the transmission of nociceptive information, then blockade by SP antagonists should result in hypoalgesia. Low doses of D-Pro\(^2\)-D-Phe\(^7\)-D-Trp\(^9\)-substance P (DPDPDT), a SP antagonist, depressed the scratching and biting behavior elicited by intrathecal SP (Piercey et al 1981, 1986) and greatly prolonged the tail withdrawal latency by noxious stimuli (Lembeck, Folkers & Donnerer 1981). Also, with intrathecal administration, this antagonist dramatically depressed the responses of mice to application capsaicin to the skin. The result was confirmed by the evidence that intrathecal [D-Pro\(^4\), Trp\(^7,9\)]SP (DPDT), another antagonist,

Recently, using three SP antagonists, DPDT, DPDPDT and [D-Pro\textsuperscript{4}, D-Trp\textsuperscript{7,9,10}]SP (DPDT-8), it was found that all selectively blocked SP-induced scratching, but not bombesin-induced scratching when all compounds were injected intrathecally in mice. However, in the tail-pinch test, of these three SP antagonists, only DPDT was found to produce a consistant antinociception, suggesting that mere blockade of a central tachykinin receptor by a SP antagonist does not invariably result in antinociceptive activity (Vaught & Post 1985). Research on SP antagonists is still at an early stage, and the specificity of the compounds has been questioned (Rodriguez et al 1983).

C. **Electrophysiological studies**

A direct comparision of the mechanism of action of SP and the transmitter released by nociceptors on dorsal horn neurones is still difficult. In spite of this obstacle, the following indirect evidence favours the idea that SP may be a transmitter in the dorsal horn.

It has been shown that slow EPSPs of neurones in the inferior mesenteric ganglion produced by stimulation of a dorsal root, but not by stimulation of a ventral root, were due to release of SP from primary afferent fibers of visceral origin (for review to see Otsuka et al 1982). With HRP techniques, it has been shown that the cell bodies of these
visceral afferents are located in the DRG and also project to the dorsal horn (Elfvin & Dalsgaard 1977). Thus, it is plausible that when such a DRG cell releases SP as a transmitter from a branch in a sympathetic ganglion, there may be a similar action at other terminals in the dorsal horn. In the rat spinal cord slice preparations, Urban & Randic (1984) found that high intensity, repetitive electrical stimulation of a dorsal root elicited slow excitatory postsynaptic potentials in about half of the dorsal horn neurones examined. Bath application of SP almost regularly produced a slow depolarization in those dorsal horn neurones that exhibited slow depolarizing potentials in response to dorsal root stimulation. Both responses were markedly depressed or abolished in the presence of SP antagonists or capsaicin. A particularly interesting recent result was that both polyclonal and monoclonal antibodies to SP significantly decreased the amplitude and duration of the slow depolarization generated in dorsal horn neurones by high intensity, repetitive dorsal root stimuli or exogenous SP application in the immature rat spinal slice preparations, suggesting that substance P mediates this slow depolarization (Randic, Ryu & Urban 1986). The presence of synapses between SP-IR containing primary afferents and dorsal horn neurones provides a structural basis for these physiological actions (Chan-palay & Palay 1977, De Lanerolle & LaMotte 1983). With in vivo experiments, Henry (1976) first reported that microelectrophoretic administration of substance P caused a
slow excitation of near half of those neurones in laminae IV-VI of the cat spinal cord which were excited by noxious heating on the skin and that excitation by substance P showed a positive correlation with excitation by intra-arterial injection (i.a.) of bradykinin which would activate peripheral nociceptors. A similar result, that SP caused excitation of dorsal horn neurones responding to i.a. bradykinin, was observed on rats (Roberts & Wright 1978). Supporting this, SP excited neurones of laminae I-III which were selectively activated by high threshold mechanical and / or thermal peripheral stimuli or by afferent volleys in Aδ and C fibres (Randic & Miletic 1977). With electrophoretic administration in the SG, SP selectively enhanced the responses of 15 of 22 neurones in laminae IV and V to peripheral noxious stimuli while depressing the activity of others (Davies & Dray 1980). These results suggest that SP is an excitatory chemical mediator of nociception in the spinal cord (Henry 1982). However, in these experiments, because of the slow conduction velocity of unmyelinated afferents there is considerable asynchrony in the arrival of impulses in the cord following a peripheral stimulus and it is thus difficult to determine whether or not a neurone, excited by SP, is excited monosynaptically by impulses in nociceptive afferent fibers. Thus to associate excitation by substance P with the postsynaptic action of a transmitter released by impulses in nociceptive primary afferent fibres is an assumption which may not be correct. In addition, if SP depolarizes a dorsal horn neurone, then responses to a
a variety of inputs (nociceptive and non-nociceptive) may be non-selectively enhanced. It does not necessarily follow that responses to afferents releasing SP will be selectively enhanced. Enhanced responses to noxious heat could equally be produced by L-glutamate. In investigating the possible selectivity of SP on nociceptive response of dorsal horn neurons, Sastry (1979) found that SP not only potentiated the intracellulary recorded responses of neurones to excitation of Aδ and C afferents of the sural nerve but also enhanced the size of EPSPs produced by impulses in group I muscle afferent fibres which are not associated with peripheral nociceptors. Thus, the proposed selective action of SP in enhancing spinal nociceptive response was shown to be incorrect. Recently, Wiesenfeld-Hallin (1986) revealed that intrathecal SP increased the amplitude of rat flexor reflexes recorded on a small filament of a muscle nerve in response to mechanical and thermal noxious stimuli. Although this reflex is complex, with many neurones and a number of transmitters, the result is still consistent with a release of SP from the central terminals of polymodal nociceptors. This view is further strengthened by the finding that the injection of SP into the intraspinal space caused mice to vigorously scratch and bite their skin in an apparent reaction to a perceived cutaneous sensation (Piercey et al 1986).

These findings collectively suggest that substance P is associated with the function of nociceptors, but the evidence is far from complete. A significant omission is the
demonstration that substance P is actually released from the central terminals of nociceptive afferent fibres. The experiments which follow have attempted to demonstrate this release.
3.2. RESULTS

3.2.1. IN VITRO experiments

Figure 5 illustrates the type of result obtained from in vitro experiments. Bound radioactivity was measured in a gamma counter and displayed by autoradiography. When the concentration of synthetic SP was zero, only $^{125}$I-labelled SP bound to the microprobes, giving a highly dense uniform autoradiographic image and high counts. No significant suppression of the binding of $[^{125}\text{I}]$SP occurred with SP concentrations of $10^{-9}$ and $10^{-11}$M. With SP at $10^{-7}$ and $10^{-5}$M, however, nearly complete suppression of the binding of the radiolabelled peptide was observed as shown by faint autoradiographic images and by counts close to background.

Parallel assays were performed with neurokinin A which occurs in comparable amounts to SP in mammalian tissue (Ogawa, Kamanazqua & Kimura 1985). This peptide was not detected by antibody microprobes at a concentration of $10^{-5}$M.

3.2.2. The Localization of the Sites of SP-IR Release by Periperal Nerve Stimulation

The experiments were performed on 5 cats. SP-IR release from specific areas of the spinal cord was estimated by measuring a zone of inhibition of the binding of $[^{125}\text{I}]$SP on autoradiographs of probes. Thus, such Inhibition of $[^{125}\text{I}]$SP
Fig. 5. In vitro assay of SP with antibody microprobes. Each antibody microprobe was preincubated for 30 minutes in a solution of SP of concentration indicated on the left and then in a solution of \([^{125}\text{I}]\text{SP}\) (1500 cpm/µl) for 24 h. The counts per millimetre (on the right) are the means of 4 microprobes. The high counts for SP, \(10^{-9}\), resulted from microprobes of slightly larger diameter being used. The autoradiographs were exposed for 4 days and the illustrations are photographic enlargements taken directly from X-ray film.
<table>
<thead>
<tr>
<th>SUBSTANCE P</th>
<th>COUNTS PER MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONCENTRATION</td>
<td>± SEM</td>
</tr>
<tr>
<td>0</td>
<td>128 ± 6.6</td>
</tr>
<tr>
<td>$10^{-11}$</td>
<td>126 ± 8.3</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>146 ± 5.6</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>18 ± 2.0</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>8 ± 0.8</td>
</tr>
</tbody>
</table>

MM
binding to microprobes inserted in the CNS tissue is hereafter taken as an indication of the presence of SP in the fluid around the microprobe (see discussion). Table II summarizes the results. It shows that both in the region of the SG and laminae V-VI of the dorsal horn there was a release of SP in the absence of stimulation, that this was not increased by stimulation of large myelinated afferent fibres but was greatly increased when unmyelinated afferent fibres were excited. A typical example is shown in Fig. 6. Photographic enlargements of microprobes images have been superimposed on an enlargement of a cross section of the spinal cord. Compared with no stimulation (probe A) and stimulation of Aaβ fibres (probe B), stimulation of unmyelinated fibres produced intense zones of inhibition of binding of $^{125}\text{I}]SP$ in the area of the substantia gelatinosa (probes C, D) and a small zone in lamina V (probe A). On probes A and B, there were detectable zones in the SG, probably resulting from tonic SP release. Fig. 6. also showed that prior injections of protease inhibitors (probe D) did not prevent release of SP nor did it potentiate such release.

Intense and prolonged stimulation of unmyelinated primary afferent fibers produced extensive release of SP as shown in Fig. 7. Probe D was kept in the spinal cord for 30 min during electrical stimulation of unmyelinated afferent fibers of the ipsilateral tibial nerve. In addition to release of SP-IR centered on the SG, an extension of SP into the dorsal column was clearly detected.
Table II  INHIBITION OF BINDING OF [\textsuperscript{125}I] SP MICROPROBES

<table>
<thead>
<tr>
<th>Lamina</th>
<th>Fibres stimulated</th>
<th>Extent of inhibition of binding</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Substantia gelatinosa</td>
<td>none</td>
<td>19</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>large myelinated</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>myelinated and unmyelinated</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>V–VI</td>
<td>none</td>
<td>65</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>large myelinated</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>myelinated and unmyelinated</td>
<td>31</td>
<td>20</td>
</tr>
</tbody>
</table>

The numbers in the table II are the percentage of probes used with each type of stimulus and refer to localized inhibition of binding [\textsuperscript{125}I] SP to the microprobes in regions correspondings to the SG and laminae V–VI when images of probes were superimposed on a section of the spinal cord. "0": no inhibition; "+": submaximal inhibition of binding; "++": complete inhibition.
Fig. 6. Inhibition of $[^{125}\text{I}]$ SP to antibody microprobes placed in the dorsal horn.

The autoradiographs of each antibody microprobe resulted from 3 days exposure on X-ray film. As in Fig. 5, enlargements were made of microprobe images and these have been superimposed on an enlargement of a 50 μm unstained cross section of spinal cord. The probes on the left resulted from no stimulation (A, 20 min in the cord) and with electrical stimulation of only the large myelinated afferent fibres of the ipsilateral tibial nerve (B, 30 min). The stimulus was 1.5 x threshold using a continuous tetanus of 31 Hz, 0.3 ms pulses. The microprobes on the right resulted from electrical stimulation adequate to excite unmyelinated fibres of the ipsilateral tibial nerve (0.3 ms pulses continuous tetanus 31 Hz, stimulus strength greater than 100 x threshold). C, 20 min in the cord; D, 30 min. Microprobe D was inserted 3 mm into the spinal cord. Just prior to the insertion of probe D, 0.5 μl of protease inhibitors was microinjected in the SG region (1 mm from the cord surface). Calibration bar : 1 mm.
Fig. 7. The effects of A and C fibre stimulation on antibody microprobes inserted in the dorsal horn.

The microprobes on the left were 20 (A) and 30 (B) min in the spinal cord during stimulation of only the large myelinated afferent fibres of the ipsilateral tibial nerve (trains of 3, 0.3 ms, pulses, 310 Hz, repeated at 2 Hz, stimulus strength 1.25 X threshold). Those on the right were 10 (C) and 30 (D) min in the spinal cord during electrical stimulation of the ipsilateral tibial nerve adequate to excite unmyelinated primary afferent fibres (stimulus parameters as above except for a stimulus strength of 250 X threshold). Microprobe B was inserted 3 mm into the spinal cord. Calibration bar : 1 mm.
3.2.3. Noxious Heat Stimulation and Release of SP-IR in the Spinal Cord

Results were obtained from 105 microprobes. Of these, 33 were controls with the lower limb not immersed in water (room temperature at 22°C), 27 with the lower limb in water at 37°C, 9 with the limb in water at 50°C and 36 with the limb in water at 52°C.

Extracellular recordings of multi-unit activity at 2 mm from the surface (lamina V) were obtained from probes before and during immersion of the limb in hot water. Fig.8B shows the responses of a neurone in lamina V by touching the skin of the fourth and fifth digits of the ipsilateral hind limb, by placing the limbs in hot water 50°C and the sustained firing which followed such immersion. The increase in firing produced by hot water returned to pre-immersion levels with each period of cooling indicating that any possible tissue damage was insufficient to produce sustained firing of nociceptors.

Immersion of the limb in warm water (37°C) did not increase release of SP over that with the limb in room air (23°C), but release was significantly increased by immersion in water at 50°C and further increased by water at 52°C. Examples of these effects are shown in Fig.8A in which microprobe images have been superimposed on a spinal cord section. It is clear that SP-IR release was detected in the region of the SG with immersing the ipsilateral hind paw in water at 50°C and 52°C (probes 2 and 4). Fig.8A also shows
Fig. 8. Release of SP and firing of a dorsal horn neurone produced by noxious heat stimulation.

A. Inhibition of binding of $[^{125}\text{I}]$SP to antibody microprobes with noxious heating of the skin of the ipsilateral hindlimb. Photographic enlargements of X-ray images of microprobes have been superimposed on a similarly enlarged section of the lumbar spinal cord. The location of a deposit of pontamine sky blue was the basis for positioning the tips of the microprobes.

Microprobe 1 was 30 minutes in the spinal cord with the ipsilateral hind paw in water at 37°C.

Microprobe 2 was 20 minutes in the spinal cord with the ipsilateral hind paw in water at 50°C.

Microprobe 3 was 30 minutes in the spinal cord with the ipsilateral hind paw in water at 52°C.

Microprobe 4 was 30 minutes in the spinal cord with limb in air at 23°C.

As the autoradiographic images of microprobes show considerable scattering the actual dimensions of one (5) are shown diagrammatically to the right of the spinal cord. Actual tip sizes were 5-10 µm. Calibration bar = 1 mm.

B. Ratemeter records of multi-unit firing recorded with microprobe 2 (depth : 2 mm from the cord surface). Note the increases produced by touching the skin of the lateral hind limb digits 4 and 5, the large increase produced by handing the limb and placing it in water at 50°C and the sustained firing which followed.
that the amounts of SP released depended on the temperatures of the water bath and the duration of immersion. With water at 50°C for 20 min; the zone of inhibition of binding was centered on the SG (probe 2) but with the more intense noxious thermal stimulus applied for a longer time (52°C for 30 min), apparent massive release extended over several spinal laminae (probe 3). Histograms of the effect of noxious heat in causing release of SP centered on the SG are shown in Fig.9.

These histograms clearly show:

A, Release of SP-IR by noxious heat was consistently detected in the region of the SG;

B, Release with probes in the spinal cord for a longer times (20-30 min) was much higher than that with immersion for shorter times (10-15 min);

C, For the same duration of immersion, release of SP-IR for water at 52°C was much higher than that for water at 50°C.

Additionally, release of SP-IR centered on lamina V was very small compared with that centered on the SG. Table III indicates release of SP in lamina V with immersion for 20-30 min with each type of stimulus. There was negligible release centered on lamina IV (although in some cases there was considerable overflow from the SG).

An interesting additional finding was that release of SP was sometimes detected at the surface of the cord at the sites of removal of the spinal pia which is normally done to permit entry of glass micropipettes into the spinal cord.
A PROBES 10 - 15 MIN IN SPINAL CORD

CONTROL  WATER 37°C  WATER 50°C  WATER 52°C

N=9  N=15  N=2  N=12

PERCENTAGE OF PROBES

100

0

PERCENTAGE OF PROBES

100

0

B PROBES 20 - 30 MIN IN SPINAL CORD

N=14  N=12  N=8  N=18

PERCENTAGE OF PROBES

100

0

Fig. 9. Histograms of the effect of noxious heat in causing inhibition of binding of $^{125}$I SP to zones of antibody microprobes corresponding to the substantia gelatinosa of the spinal cord.
Table III  NOXIOUS HEATING PRODUCES INHIBITION OF BINDING OF $[^{125}\text{I}]$ SP MICROPROBES

<table>
<thead>
<tr>
<th>Lamina</th>
<th>Noxious Heat</th>
<th>Extent of inhibition of Binding</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>none (in air)</td>
<td></td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>37°C</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>71.4</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>52°C</td>
<td>40</td>
<td>26.7</td>
</tr>
</tbody>
</table>

The numbers in the table III are the percentage of probes used with each type of stimulus. Probes were kept in the spinal cord for 20-30 min.
3.3. DISCUSSION

3.3.1. A new technique -- the antibody microprobe

To determine the involvement of various neurotransmitters in specific physiological processes, changes in the release of particular compounds have been correlated with changes in physiological events by means of several techniques (Marsden 1984). These include intracerebral and intrathecal perfusion systems (push-pull cannula and cortical cup), intracranial dialysis and voltammetry. These techniques have added extensively to our knowledge about what substances are released under physiological conditions in the central nervous system.

A novel approach, the antibody microprobe (Duggan & Hendry 1986), has been used in the present work. It employs a combination of radioimmunoassay and microelectrode recording techniques. One of its outstanding advantages is the very fine diameter of the tips of probes and there is very little damage to the brain tissue. Thus, this probe not only enables sites of SP release to be localized under physiological conditions, but can also concomitantly be used to record neuronal activity extracellularly. More importantly, such fibers should prove adequate for the detection of release of numerous other neuropeptides in the CNS in addition to SP.

3.3.2. Specific binding of substance P

An important consideration in interpreting these results
is whether the zones of inhibition of $[^{125}\text{I}]$SP binding represent focal release of SP or interference by other compounds. The conclusion that it is predominantly release of SP is based on the following evidence.

A. The in vitro results in which synthetic SP $10^{-7}$M nearly completely suppressed the binding of $[^{125}\text{I}]$SP.

B. The failure of protease inhibitors to alter the inhibition of binding; this argues against the interesting possibility that proteases are released by activity in unmyelinated primary afferent fibres, resulting in non-specific degradation of SP antibodies and thus inhibition of subsequent binding of $[^{125}\text{I}]$SP.

C. The clear focal increase in the inhibition of binding of $[^{125}\text{I}]$SP in the area where most unmyelinated primary afferent fibers terminate when these fibres were electrically stimulated. Many of these fibres contain SP (Hokfelt et al 1976, 1977b). This result contrasts with the near absence of inhibition of binding of $[^{125}\text{I}]$SP in lamina IV when large myelinated fibres were stimulated either alone or together with C-fibres. Lamina IV receives large diameter primary afferent fibres (Brown 1981) and few if any of them contain SP (Price 1985).

D. The failure of microprobes to detect neurokinin A ($10^{-5}$M) in vitro. In the rat dorsal horn the levels of SP
are approximately three-fold higher than those of neurokinin A (Ogawa, Kanazawa & Kimura 1985). Although a contribution by neurokinin A and other untested polypeptides to the inhibition of binding of $^{125}\text{I}]$SP cannot be excluded, it is unlikely to be a large component.

It is important to emphasize that the zones of inhibition of binding of $^{125}\text{I}$ SP were produced or increased by impulses in unmyelinated primary afferent fibers. Thus these zones cannot have been produced by damage to SP containing terminals in the SG since this would have produced bands of inhibition irrespective of whether peripheral nerves were stimulated or not.

3.3.3. SP release from the central terminals of primary afferent fibres in the SG

These results are the first to localize the sites of release of immunoreactive SP within the spinal cord following impulses in unmyelinated primary afferent fibres. Although it is not possible to differentiate between release of SP-IR from such afferent fibres and that from neurones of the spinal cord, there are a number of reasons to believe that release mainly came from primary afferent fibres.

B. The localizations of release and non-release of SP are parallel to the distributions of C and Aβ primary afferent terminals in the dorsal horn, respectively. Aβ primary afferent terminals are mainly located in laminae III - V (Willis & Coggeshall 1978).

C. SP-IR in the SG is heavily depleted by dorsal rhizotomy (Hokfelt et al 1975, Takahashi & Otsuka 1975, Barber et al 1979).

D. In contrast to the termination of SP containing afferent fibres mainly in the SG, SP neurones are distributed in all laminae of the dorsal horn (Kawatani, Erdman & De Groat 1985). Since after dorsal rhizotomy, only very few SP-IR fibres remain in the SG (Barber et al 1979), it seems that SP-containing terminals in the SG mainly originate from primary afferent fibres with little contribution from intrinsic SP-containing neurones. In the present experiments, release from descending fibres of the brainstem can be excluded because the cord was sectioned in all animals. It should be noted that with spinal transection peripheral nerve C fibres stimulation does not elevate blood pressure and thus, the possibility that SP release resulted from some associated indirect effect is improbable.

E. Substance P is located in the superficial layer of the trigeminal nucleus caudalis, the site of the first synaptic relays of nociceptive tooth pulp afferents
(Priestly, Somogyi & Cuello 1982). It has been found that β-endorphin and morphine reduce release of SP from slices of rat trigeminal nucleus but not release from slices of substantia nigra (Jessel & Iversen 1977). Both structures have neurones containing substance P but only the trigeminal nucleus receives primary afferents. Thus, release of SP in the trigeminal nucleus caudalis produced by tooth pulp stimulation probably comes from primary afferent fibres (Yonehara et al 1986).

F, With cultured DRG neurones, it was found that high K+ in the culture medium evoked a release of SP and that an opioid peptide inhibited this release (Mudge, Leeman & Fischbach 1979).

It is interesting that with some microprobes, massive SP release by intense noxious stimuli for long times was detectable over several spinal laminae and even in the dorsal columns. This may be the reason why release of SP can be measured in the CSF from a perfusate of the spinal cord (Yaksh et al 1980), particularly when inhibitors of degrading enzymes are used.

It is also of interest that a surface inhibition of ¹²⁵I SP binding to probes at the level of the surface of the cord was observed in the present study. It is possible that SP was released from the pia on the cord since the pia contains small calibre vessels where SP axons make contacts (Barber et al 1979, Liu-chen, Han & Moskowitz 1983, Moskowitz, Brody &
Lin-chen 1983). Its function here is unknown. In addition, an inflammatory reaction may ensue at sites of pial removal, and this may enhance the release of SP from nerves innervating blood vessels but also could result in release of enzymes which degrade antibodies attached to the probes. Further experiments are needed on this question.

In addition to evoked release, tonic release was found in some cases. In view of the evidence linking substance P to nociceptors (Jessel 1983, Salt & Hill 1983), the surgery needed to expose the lumbar spinal cord would produce firing of nociceptors in the areas innervated by the dorsal primary rami of lumbar and sacral spinal nerves, resulting in a "tonic" release of SP. This varied considerably between probes and animals and, when present, was increased by peripheral noxious stimuli.

3.3.4 Is SP released specifically by noxious mechanical stimulation?

In the present studies, SP-IR was released by impulses in thermal nociceptors. This result differs from that of Kuraishi et al (1985), in which the release of somatostatin but not SP followed radiant heating of the skin of the rabbit. The latter was released by mechanical stimuli. In explaining this difference two possibilities must be considered:

(a) The depth of the push-pull cannula in the dorsal horn was not stated by Kuraishi et al (1985). A rather big cannula (600 µm diameter) would certainly damage the SG if
inserted into lamina IV/V.

(b) More likely, however is the use of inadequate skin temperatures for peripheral noxious stimulation. It was stated that a subcutaneous thermocouple showed that temperatures during heating rose to a maximum of 48.5°C and were above 44°C for 11.5 of the 20 minute period of heating. Compared with the present experiments, these temperatures are not high and may not have produced sufficient release of SP to be detected with a push-pull callula.

Additionally, Kuraishi et al (1985) believed their results were consistent with different populations of DRG cells containing either substance P and somatostatin, as originally reported for the rat by Hokfelt et al (1976). This may not be true for all vertebrates, however, as in the cat it has been found that 83% of DRG neurones which contained somatostatin-IR also contained SP-IR (Leah, Cameron & Snow 1985). Another difficulty with the results of Kuraishi et al is what kind of nociceptors was stimulated? It is known that the main C fibre afferent input is from polymodal nociceptors responding to both noxious mechanical and noxious thermal stimuli and that polymodal nociceptors account for around 80% of fibers in the skin (Lynn & Hunt 1984). The peptide(s) released from polymodal nociceptors should be equally released by both mechanical and thermal noxious stimuli and this was not found by Kuraishi et al (1985). If it is true that a specific peptide is associated with a particular stimulus, it implies that the major (polymodal) nociceceptor releases neither SP nor somatostatin. Of interest
is finding of Kuraishi et al that somatostatin was released by these lower skin temperatures, suggesting a role in the transmission of impulses from thermal receptors.

More recently, an indirect approach led Wiesenfeld-Hallin (1986) to propose that both SP and somatostatin are released by thermal nociceptors. The flexor reflex of decerebrate rats to thermal peripheral stimuli was increased by both SP and somatostatin applied topically to the spinal cord. Reflexes to mechanical stimuli were increased only by SP. Although this reflex may be complex, with receptors for the two peptides on many of the neurones involved, the result is particularly interesting in implying a release of both SP and somatostatin by noxious thermal stimuli. Also, additional support is provided by Go and Yaksh's report in which a mild release of SP was produced by a thermal stimulus (55°C) to the skin in the cat (1986).

3.3.5. The significance of the present results for the status of SP as a transmitter released from nociceptive primary afferent fibres

As evidence outlined in the introduction, ever since SP was proposed as a sensory transmitter (Lembeck 1953), a correlation has been repeatedly made between substance P and nociceptive primary afferents fibres. SP-IR is mainly located in laminae I and II of the dorsal horn and associated with large dense-core vesicles in nerve terminals (Barber et al 1979, Bresnahan, Ho & Beattie 1984). SP has been released from sensory terminals in the dorsal horn in vitro (Otsuka et
al 1982) and in vivo (Yaksh et al 1980, this thesis). The slow, synaptically mediated depolarization evoked by repetitive stimulation of a dorsal root is mimicked by administration of SP and blocked by SP antagonists (Urban & Randic 1984). Thus, SP fulfills several of the major criteria for transmitter identification.

The results presented here on SP release detected by antibody microprobes provide important support for the idea that SP may be a transmitter in synaptic transmission from primary afferent fibres that mediate nociception (Jessel & Iversen 1977, Nicoll, Schenker & Leeman 1980, Jessell 1982).

Importantly, what is clear from the present results is that the SP-IR is released mainly in the SG when cutaneous nociceptors are excited physiologically by noxious heat to the skin or when unmyelinated afferent fibres are stimulated electrically. The SG is a strategic site in the processing of sensory signals in the dorsal horn. In this region, anatomically, there is a close intermingling of SP, opioid peptides (Hokfelt 1977a), opioid receptors (LaMotte, Pert & Snyder 1977) and primary unmyelinated afferent fibres conveying nociceptive signals (Nagy & Hunt 1983, Sugiura, Lee & Perl 1986). Physiologically, the SG probably plays a leading role in control of nociceptive transmission in the spinal cord (Duggan, Hall & Headley 1977, for review see Wall 1980). Although the slow time-course of action of SP differs from that of excitant amino acids such as L-glutamate, intracellular recording from dorsal horn neurones of rat spinal cord slices has revealed the presence of both fast and
slow depolarizing postsynaptic potentials from stimulation of primary afferent fibres (Urban & Randic 1984), suggesting that primary sensory neurones probably release both fast and slow acting excitatory transmitters. More than a dozen neuropeptides and other compounds have been identified within mammalian DRG neurones and some of them co-exist in the same neurone (Jessel & Dodd 1986). Thus, it is possible that an unidentified fast acting transmitter is released together with SP from a single primary afferent terminal in producing transmission of nociceptive information.

Early studies found that ATP is released from the peripheral terminals of unmyelinated sensory neurones, suggesting that release of ATP might also occur from central sensory terminals (Holton 1959). In DRG neurones grown in co-culture with dorsal horn neurones, application of ATP produced a rapid and marked depolarization of approximately 25% of dorsal horn neurones tested (Jahr & Jessel 1983). This compound activated both nociceptive and non-nociceptive neurones in the dorsal horn of the spinal cord (Fyfe & Perl 1984) and the medulla (Salt & Hill 1983). These results, although preliminary, suggest that ATP may be released as a fast acting sensory transmitter in the spinal cord. However, many spinal neurones which receive primary afferents are insensitive to ATP (Curtis, Phillis & Watkins 1961, Jahr & Jessel 1983). As a result, ATP may not be the only fast acting excitatory transmitter released from DRG neurones. More recently, Schouenborg & Sjolund (1986) have demonstrated that γ-D-glutamylglycine (γ-DGG), a relatively selective
antagonist of excitant amino acids, inhibited, dose-dependently, nociceptive C fibre-evoked field potentials and discharges of single nociceptive neurones in the rat spinal cord. Such a result suggests that the transmitter substance released from nociceptive afferent fibres and responsible for fast EPSPs may be L-glutamate and/or L-aspartate.

The detection of L-glutamate and L-aspartate release in a manner analogous to that of substance P is currently not possible. However, it is an important question to determine whether other compounds are released in addition to SP in the dorsal horn of spinal cord when peripheral nociceptors are activated physiologically.
CHAPTER 4  SEGMENTAL CONTROL OF NOCICEPTIVE TRANSMISSION

4.1. INTRODUCTION

The dorsal horn of the spinal cord is an important site for the control of nociceptive information (Willis & Coggeshall 1978). Following the isolation of the opioid peptides, methionine (Met-) and Leucine (Leu-) enkephalin, from brain (Hughes et al 1975), there have been many investigations of the role of such opioid peptides in controlling the transmission of impulses related to nociception. Increasing evidence has indicated that opioid peptides have a number of roles in the central nervous system. This chapter will mainly deal with the actions of opioid peptides on the spinal cord, particularly on nociception.

4.1.1. The Superficial Dorsal Horn

The gray matter of the dorsal horn has been subdivided into 6 laminae, based on cytoarchitectonics, by Rexed (1952). This laminar subdivision is still useful, but extensive studies has subsequently given more detailed knowledge of the termination of primary afferent fibres, local circuit neurones, descending terminals from brainstem, etc, in these various laminae.

A. Termination of primary afferent fibres

The development of the technique of labelling single
neurones and axons by injection of the enzyme horseradish peroxidase (HRP) (Snow, Rose & Brown 1976, Cullheim & Kellerth 1976, Jankowska, Rastad & Westman 1976, Light & Durkovic 1976, Light & Perl 1976), enabled a comparison to be made of the function and the morphology of the central terminals of a particular type of afferent fibres. Generally, the larger the fiber diameter, the deeper are its terminals in the cord. The large myelinated cutaneous afferent fibres characterized as low threshold mechanoreceptors, initially travel ventrally through the dorsal horn but turn dorsally when they reach deep laminae and end predominantly in laminae III, IV and V (Brown, Rose & Snow 1978, Brown 1981, Dodd, Solter & Jessell 1984, Woolf & Fitzgerald 1986).

(a) Small myelinated afferent fibres from mechanical nociceptors

With the HRP label technique, the central terminations of fine myelinated afferent fibres from physiological identified sensory units has been traced in different species (Light & Perl 1979, Rethelyi, Light & Perl 1982). Most high threshold mechanical fibres (Aδ) terminate at the border between lamina I and the outer part of the SG. A second site concentrates in lamina V, particularly the reticulated portion at the lateral border of the dorsal horn. In addition, many high threshold mechanical fibers terminate in lamina X.

(b) Unmyelinated afferent fibres from nociceptors

The majority of primary afferent C fibres are
polymodal high-threshold nociceptors (Bessou & Perl 1969, Lynn & Carpenter 1982). Until recently the extremely fine diameter of unmyelinated fibres (less than 1 μm) has prevented direct examination of their the terminations. However, the combined evidence from Golgi staining, from transport of radiolabelled compounds, degeneration and neurochemical studies, has indirectly indicated that primary unmyelinated afferent fibres end predominantly in lamina II (SG), with a lesser projection to lamina I (Sweet & Woolf 1985, for review see Brown 1982, Duber & Bennet 1983, Perl 1984, Hunt & Rossi 1985). More recently, Perl's group has directly demonstrated that the superficial layers of the spinal dorsal horn, especially the SG, appear to be the main projection zones for unmyelinated primary afferent fibers from skin (Sugiura, Lee & Perl 1986). These investigators used a plant lectin, phaseolus vulgaris leukoagglutinin (PHA-L), which readily fills distant processes by orthograde transport. This compound was injected intracellularly into dorsal root ganglion cells by electrophoresis as an immunocytochemical marker. The majority (more than 80%) of labelled C-fiber units were functionally characterized as nociceptors (mechanical, polymodal and cold nociceptors). The central branches of C fibre units from the skin arborized and terminated mainly in the superficial layers of the spinal dorsal horn. This is consistent with electrophysiological results (McMahon & Wall 1985), using intraspinal antidromic stimulation, when it was found that sural C afferent fibres terminated in lamina I and outer lamina II whereas
gastrocnemius (muscle nerve) C afferent fibres terminated in inner lamina II. However, the location of terminations of C afferent fibres from visceral and subcutaneous tissues is still unclear. Ultrastructurally, C fibers terminate in glomerular complexes in which axo-dendritic, axo-axonic and dendro-dendritic synapses have been described (Gobel et al 1982, Riberiro-Da-Silva & Coimbra 1982).

B. Neurones related to nociceptive transmission

A number of electrophysiological studies have shown that neurones responding to impulses in nociceptive afferent fibres occur in many spinal laminae. Noxious specific (NS) neurones are excited only by high-threshold or noxious peripheral stimulation. Wide dynamic range (WDR)(also called multireceptive or convergent) neurones respond to impulses in both innocuous low-threshold and noxious high-threshold afferent fibres (Willis & Coggeshall 1978).

Lamina I neurones: the large marginal neurones have relatively long and unbranched dendrites which are contained largely within lamina I but a few distribute in lamina II. Many lamina I neurones send their axons into Lissauer's tract (Szentagothai 1964) and give rise to ascending projections (Scheibel & Scheibel 1968). Some lamina I neurones project directly to the contralateral thalamus (Applebaum et al 1975, Carstens & Trevino 1978, Craig & Burton 1981) through the dorsolateral funiculus (McMahon & Wall 1983, Apkarin, Stevens & Hodge 1985).
The function of lamina I neurones of the cat was first studied by Perl's group (Christensen & Perl 1970, Kumazawa & Perl 1976, 1978) which described three types of neurone excited by impulses in peripheral afferent fibres. The first group of neurones were excited predominantly by the small myelinated fibers (Aδ) derived from cutaneous mechanical nociceptors. The second group receive a specifically nociceptive input from unmyelinated afferent fibers of polymodal nociceptors. The last group responded to innocuous temperature changes of the skin. WDR neurones, however, have been found in this lamina (Bennett et al 1981).

**The substantia gelatinosa (SG):** this layer is located just ventral to lamina I and is Rexed's lamina II (1952, 1954). It is readily distinguished from other spinal laminae by its closely packed small neurones and the absence of myelinated fibres. The morphology and functional roles of the SG have been studied extensively (for review see Willis & Coggeshall 1978, Cervero & Iggo 1980, Wall 1980, Dubner & Bennett 1983). In brief, the SG contains four components: primary Aδ and C afferent fibres, dendrites from large neurones of laminae IV and V (Szentagothai 1964, Perl 1984), descending fibres from the brainstem (Ruda & Gobel 1980) and intrinsic neurones, of which there are at least five morphological types (Gobel 1979).

The stalked cell and the islet cell are two principal types of neurones in the SG. The stalked neurones are found at the border between laminae I and II and project dorsally.
to lamina I, and ventrally to laminae II-IV. Their dendrites receive synapses also from primary afferent terminals and probably from descending fibres (Gobel et al 1982). The islet neurone perikarya are located throughout the SG and have a limited axonal arborization distributed profusely within their dendritic tree. Their dendrites synapse with primary afferent fibers, dendrites from other neurones (possible stalked neurones) and descending terminals (Gobel et al 1982, Perl 1984).

The SG is divided into two sub-laminae, IIa (superficial) and IIb (deeper) (Gobel 1979). Using the intracellular HRP technique, Bennett et al (1979, 1980) found that the responses of stalked neurones and lamina IIa islet neurones were comparable to the responses of noxious specific (NS) and WDR neurones, respectively, while lamina IIb islet neurones were excited only by impulses in low threshold mechanoreceptive primary afferent fibers (Kumazawa & Perl 1976, Price et al 1979, Bennett et al 1980). Many neurones respond less effectively to repeated stimuli whereas others have prolonged after-discharges lasting seconds or minutes following a single stimulus (Light, Trevino & Perl 1979, Wall, Merrill & Yaksh 1979, Bennett et al 1980).

**Lamina IV neurones**: the nucleus proprius of the spinal cord consists of laminae III and IV, a relatively thick layer which extends across the dorsal horn. Retrograde HRP studies have shown that the great majority of spinocervical tract (SCT) neurones are in lamina IV (Craig 1976, Cervero, Molony
& Iggo 1977, Brown 1982). In addition, some neurones belong to the spinothalamic tract system and propriospinal system. In general, lamina IV neurones receive a predominantly peripheral excitation from low-threshold mechanoreceptors having large myelinated afferent fibres. They are excited by hair movement, touch and stimulation of tactile domes (Willis & Coggeshall 1978). But a fraction of the group contributing to the second-order dorsal column projection show maximal responses to noxious stimulation (Brown 1981). Thus, WDR neurones are also present in this layer.

Lamina V neurones: the most common type of neurone in this layer is the WDR neurone (Wall 1967, for review see Willis & Coggeshall 1978). Many have the following morphological features: (i) large cell bodies; (ii) extensive dendritic spread in all directions; and (iii) axons ascending in the contralateral ventral white matter (Scheibel & Scheibel 1968, Brown 1981, Ritz & Greenspan 1985). Some noxious specific neurones (NS) exist in this lamina. Compared with the WDR neurones, these NS neurones have smaller cell bodies and a similar dendritic spread (Ritz & Greenspan 1985).

4.1.2. Opioid Peptides and Segmental Inhibition

A. Opioid peptides in the spinal cord

Numerous opioid peptides have been identified since met- and leu-enkephalin were discovered (Hughes et al 1975). All belong to three peptide families:
i, pro-opiomelanocortin (the precursor of \(\beta\)-endorphin);
ii, pro-enkephalin A (the precursor of \(\text{met}^5\)-enkephalin)
iii, pro-enkephalin B (the precursor of the dynorphins and \(\alpha\)-neo-endorphin and \(\text{leu}^5\)-enkephalin)

Of these families, the latter two are found in the spinal cord (for review see Hollet 1986).

(a) **Opioid receptors and ligands**

The notion that there are several subclasses of opiate receptor was first formulated by Martin (1967), on the basis of the differential pharmacological effects of morphine, ketocyclazocine and N-allyl-normetazocine in the chronic spinal dog. In the past decade, it has become clear from numerous studies that there are at least three important receptors subtypes: \(\mu\), \(\delta\) and \(\kappa\) (for review see Kosterlitz 1985, Hollet 1986).

A full complement of opioid receptors (\(\mu\), \(\delta\) and \(\kappa\)) has been demonstrated in the spinal cord by pharmacological tests (Wood, Rackham & Richard 1981, Tung & Yaksh 1982, Hylden & Wilcox 1983). By binding studies in vitro, a higher density of \(\kappa\) sites than, \(\mu\) and a small number of \(\delta\) sites, were found in the rat (Gouarderes, Audigier & Cros 1982) and human spinal cord (Czlonkowski et al 1983). However, the markedly different distributions of opioid peptide sub-types in the various levels of the spinal cord were revealed by autoradiography (Gouarderes, Quirion & Cros 1984). Mu binding sites are highly concentrated in the dorsal horn, almost evenly along the whole spinal cord. Delta binding
sites are found mainly in cervical and thoracic segments, and kappa receptors are located principally in the lumbo-sacral spinal cord in the rat.

On the basis of the binding of radiolabelled etorphine, a ligand for all three receptor subtypes, a high density of binding sites was found in laminae I and II of the dorsal horn (Atweh & Kuhar 1977, Simon & Hiller 1978) and on fibres of the medial part of the proximal dorsal root (Walmsley 1983). As mentioned above, the superficial dorsal horn preferentially receives small myelinated Aδ afferent fibres and unmyelinated C afferent fibres which convey nociceptive inputs. The deeper laminae of the dorsal horn which receive mainly large myelinated afferent fibers have few opioid binding sites. The findings that dorsal root section in the monkey resulted in a loss of opioid binding in the spinal dorsal horn, and that opioid receptors were located on neurites of DRG cells in culture preparation (Hiller et al 1978), suggest that opioid receptors are located at least in part presynaptically on terminals of primary afferent fibers (LaMotte, Pert & Snyder 1976). Supporting this proposal, morphine administered microelectrophoretically in the SG, but not near cell bodies, selectively suppressed transmission of nociceptive impulses in the dorsal horn (Duggan, Hall & Headley 1977). However, in view of the presence of residual opioid binding after extensive dorsal rhizotomies (Lamotte, Pert & Snyder 1976), the existence of postsynaptic opioid receptors in the spinal dorsal horn cannot be excluded.
Since the discovery of opioid receptors, there has been extensive investigations of the endogenous ligands for the different types of receptor. As far as is known, none of the endogenous opioid peptides binds at only one of the µ, δ or κ-sites. However, there appears to be a high affinity between enkephalins and δ receptors (Lord et al 1977, McKnight et al 1984), and between dynorphins / neo-endorphins and κ receptor (Oka et al 1981, Chavkin, James & Goldstein 1982, Paterson, Robson & Kosterlitz 1983). In contrast, no endogenous opioid peptide has very high selectivity for µ receptors, and also no isolated tissue preparation has been found to have only µ receptors, in contrast to those with δ and κ receptors. Recently metorphamide, a endogenous novel and large opioid peptide, has been found to have a high affinity for µ sites, although still binding to κ sites and with a very low affinity for δ sites (Weber et al 1983). With pharmacological tests, dermorphin, another novel opioid peptide found in amphibian skin, was considered as possessing all of the characteristics of a µ receptor agonist (Stevens & Yaksh 1986).

(b) Distribution of opioid peptides in the spinal cord

(i) Terminals: A high density of both enkephalin-(ENK) and dynorphin-immunoreactive fibres was present in laminae I, II and V of the dorsal horn, as well as lamina X, in the rat (Petrusz, Merchenthaler & maderdruit 1985) and cat (Glazer & Basbaum 1981, Przewlocki et al 1983). Compared with to ENK, dynophin was relatively restricted to laminae I
and V of cat dorsal horn. A few dynorphin-terminals were found in lamina IIa and no dynorphin was located in lamina IIb, whereas the heaviest distribution of ENK-terminals was found in this latter region (Cruz & Basbaum 1985). Additionally, ENK fibres in laminae VIII and IX were located close to motoneurones (Petrusz, Merchenthaler & Madrdrut 1985), and there were minimal dynorphin fibres in the ventral horn (Cruz & Basbaum 1985). ENK terminals in the dorsal horn are commonly considered to be those of local interneurones, since transection of the spinal cord or rhizotomy resulted in no significant reduction of these ENK terminals (Seybold & Elde 1980, Hunt et al 1981). In contrast, a recent report found that a significant proportion of the sacral spinal cord dynorphin IR-terminals was derived from primary afferent fibers (Basbaum, Cruz & Weber 1986).

(ii) ENK-containing neurones: The distribution of ENK IR-neurones is similar to that of ENK-terminals (Glazer & Basbaum 1981, Bennett et al 1982, Hunt, Nagy & Ninkovic 1982). The largest number of ENK-containing neurones occur in lamina III (Cruz & Basbaum 1985). Lamina I neurones with a short local axon (Bennett et al 1981), and some stalked and islet neurones of lamina II have been identified as ENK-containing cells (Bennett et al 1982). Since unmyelinated primary afferent fibres terminate in the superficial dorsal horn, these ENK containing neurones are ideally situated to control spinal nociceptive transmission. ENK-containing lamina I neurones and stalked neurones probably synapse on lamina I projection neurones.
are likely to synapse on the dendrites of stalked neurones and indirectly influence lamina I projection neurones (Gobel et al 1982, for review see Dubner et al 1984). Using the combined techniques of retrograde HRP and enkephalin immunocytochemistry, it has been demonstrated that the thalamic projection neurones receiving ENK-containing axons in lamina I, and those in lamina V, represent roughly 30% and more than 50% of the total number of projection neurones, respectively (Ruda, Coffield & Dubner 1984). Ultrastructural studies showed that almost all ENK synapses were axodendritic or axosomatic (Hunt, Kelly & Emson 1980, Aronin et al 1981, Glazer & Basbaum 1983, LaMotte & de Lanerolle 1983, Ruda, Coffield & Dubner 1984).

(iii) Dynorphin-containing neurones : Cruz & Basbaum (1985) have identified important differences in the distribution of dynorphin and enkephalin neurones in the cat. They found that over 77% of dynorphin-containing neurones were distributed between laminae I and II and 10.6% in lamina V. In contrast to ENK-containing neurones, there were almost no dynorphin-containing neurones in lamina III.

(c) Degradation of opioid peptides and enzyme inhibitors

Since Hughes (1975) found that carboxypeptidase A and leucine-aminopeptidase destroyed the activity of enkephalins during the isolation of these peptides, peptidases and their inhibitors have been widely studied (for review see Hughes 1983, Schwanty et al 1981, 1983, 1985). Enkephalin activity at the opiate receptor level appears to be regulated by
enzymic degradation. There is no evidence for uptake as an important process in degradation of opioid peptides. As a consequence, inhibitors of these enzymes may be useful pharmacocloal tools with which to potentiate the action of enkephalins.

(i) **Degradation**: aminopeptidase, dipeptidylaminopeptidase, dipeptidylcarboxypeptidase (enkephalinase), angiotensin-converting enzyme (ACE) and carboxypeptidase A can inactivate the enkephalins through cleaving various bonds at different positions (Fig.10) (Hambrock et al 1976, Knight & Klee 1977, Malfroy et al 1978, 1979, Gorenstein and Snyder 1979, for review see Frederickson 1984). Among these peptidases, aminopeptidase and dipeptidylcarboxypeptidase (enkephalinase) seem to be the most important. For the remainder, there is no evidence for a role in synaptic inactivation. Fig.10 summarizes their potential roles. The cleavage of any bond will generate products with little or no activity at opiate receptors.

(ii) **Peptidases inhibitors and analgesia**: various inhibitors of these enzymes have been used in attempts to protect the cleavage of ENK bonds. Such inhibitors could reveal physiological events involving these opioid peptides, particularly in regard to nociception. Four inhibitors will be discussed.

- **D-phenylalanine (DPA)** is an inhibitor of carboxypeptidase A (Hartsuck & Lipscomb 1971). By hot plate
Fig. 10. Modes of cleavage of enkephalins by enzymes.
testing, the i.p. administration of D-phenylalanine produced analgesia in mice (Eherenpreis et al 1979, Alleva, Castellano & Oliverio 1980, Filibeck, Castellano & Oliverio 1981). This compound was tested, along with other inhibitors of carboxypeptidase A on the belief that they would decrease the degradation rate of opioid peptides within the CNS. The finding that naloxone (20mg/kg i.p.) reversed or prevented analgesia from DPA may indicate that the latter acts through enhancing the action of opioid peptides (Ehrenpreis et al 1979), but the effects of naloxone alone in these experiments were not reported. DPA has been found to give some relief to patients with chronic pain (Ehrenpreis et al 1980, Balagot et al 1983, Budd 1983, Hoda, Kitadi & Hosaka 1983), and to potentiate acupuncture analgesia in both man (Takeshige et al 1983) and mice (Cheng & Pomeranz 1980, Han et al 1981).

Thiorphan is a highly potent metal-chelating "enkephalinase" inhibitor (Roques et al 1980). The evidence that low concentrations of thiorphan selectively protected Met-enkephalin released from brain slices probably indicated the association of enkephalinase with the synaptic activity of an endogenous opioid peptide (Pateyet et al 1981). Indeed the presence of enkephalinases probably results in the very weakly analgesic effect when enkephalins are administered centrally (Graf 1976, Yaksh et al 1977). A naloxone reversible analgesic effect was observed following i.c.v. injection of thiorphan using the mice hot plate test (Roques et al 1980, Zhang Yang & Costa 1982) and by i.t. injection using the rat hot plate and tail flick test (Yaksh & Harty
1982). Also, thiorphan potentiated electro-acupuncture analgesia (Jin, Zhou & Han 1986).

The third compound **bestatin** is an aminopeptidase inhibitor. With i.t administration of this inhibitor, a potentiation was seen of both ENK-induced analgesia and antinociceptive activity in mice (Carenzi, Frigeni & Della Bella 1981, Chaillet et al 1983, Costentin et al 1986), and acupuncture-induced analgesia in rabbits (Jin, Zhou & Han 1986).

Finally, kelatorphan, a very potent inhibitor of multiple ENK degrading enzymes, was synthesized by Roques's group (Fournier-Zaluski et al 1984). In rat striatal slices, kelatorphan almost abolished the formation of 3 metabolites of Met-ENK resulting from aminopeptidase, enkephalinase and dipeptidylaminopeptidase activity (Waksman et al 1985). Administration of kelatorphan (i.c.v.) to mice produced analgesia and potentiated ENK-induced analgesia by a factor of 50,000 (Fournier-Zaluski et al 1984).

Fig.11 depicts these inhibitors and their corresponding peptidases.

To date, all results of inhibitor-induced antinociception were derived from behavioral experiments with i.c.v. and i.t administration of peptidase inhibitors. To further investigate their possible effect in the spinal cord, an electrophysiological study is appropriate. Recently, Waksman et al (1985) have demonstrated that enkephalinase and opioid binding sites are unevenly distributed in the CNS, but
Fig. 11. Inhibitors and their corresponding enzymes.
relatively high concentrations occur in the substantia gelatinosa of the spinal cord. Thus the SG of the spinal cord is an ideal site to investigate interactions of peptidase inhibitors with endogenous and exogenous enkephalins under in vivo conditions. The studies described in this thesis will deal with the question as to whether peptidase inhibitors potentiate ENK-induced inhibition of nociceptive responses of dorsal horn neurones.

(d) Effects of opioid peptides on spinal neurones

On the basis of the presence of multiple opioid receptors in the spinal cord, the interaction between ligands and receptors might be expected to result in modulation of spinal nociceptive transmission. During the last decade, the antinociceptive action of opiates and opioid peptides at spinal level have been investigated extensively (Martin et al 1976, Tyers 1980, Han & Xie 1982, for review see Yaksh 1981, Yaksh & Nouihed 1985). Intrathecal administration of \( \mu \), \( \delta \) and \( \kappa \) opioid ligands produces antinociception in mice and rats, indicating that multiple receptors are involved in spinally mediated antinociception (Przewlocki et al 1983, Satoh et al 1983, Schmauss et al 1983). More interestingly, a recent study from Yaksh's group suggest that the activity of three opioid receptors results in a selective modulation of the response of the animal to differing noxious stimuli (Schmauss & Yaksh 1984). They found that i.t. administration of a \( \mu \) ligand produced a dose-dependent inhibition of both noxious cutaneous thermal and visceral chemical stimuli in
the rat. In contrast, δ and κ ligands only depressed thermal and visceral chemical responses, respectively.

The electrophysiology of opioids has been extensively studied (for review see Duggan & North 1985). At the spinal level, the findings demonstrate that the excitation of neurones of the dorsal horn by impulses in unmyelinated primary afferent fibres is depressed by the administration of an opiate either systemically (Le Bars et al 1975, Jurna & Grossman 1976, Zieglgansberger & Bayerl 1976, Einspahr & Piercey 1980) or microelectrophoretically in the SG (Duggan, Hall & Headley 1977a). When natural cutaneous stimuli are used, nociceptive heat responses of deeper neurones in lamina IV/V are selectively inhibited by microelectrophoretic administration of morphine in the SG, but little, if any, effect on responses to deflection of hairs is observed. When morphine was ejected near cell bodies, variable effects have been seen (Willcockson et al 1986). Whereas some groups have observed naloxone reversible effects suggestive of postsynaptic actions of opiates (Calvillo, Hendry & Neuman 1974, Zieglgansberger & Bayerl 1976), other investigators have observed effects by opiates not reversed by naloxone and hence regarded as of little relevance to the effects of systemic opiates (Duggan, Davies & Hall 1976, Dostrovsky & Pomeranz 1976, Duggan, Johnson & morton 1981). Since unmyelinated primary afferent fibres (Light & Perl 1977), and a high concentration of opioid binding sites (Atweh & Kuhar 1977) are located in the SG, it has been proposed that the selective inhibition of opiates on nociceptive transmission
probably results from a reduced release of transmitter from
the terminals of unmyelinated primary afferent. After the
discovery of enkephalins, their effects on neurons of the
spinal dorsal horn has been investigated by several
laboratories (Duggan, Hall & Headley 1977b, Randic & Miletic
Compared with morphine, there is an important difference in
the effect of enkephalins on dorsal horn neurones. When
microelectrophoretically administered in the SG, Met-
enkephalinamide selectively depressed the activation of
neurones in laminae IV and V by noxious cutaneous stimuli
whereas relatively non-selective inhibition of these
responses occurred when this peptide was ejected near cell
bodies (Duggan, Hall & Headley 1977b, Duggan, Johnson & Morton
1981). A similar result was obtained when [D-Ala², MePhe⁴,
Gly-01]enkephalin (DAGO) was administrated
microelectrophoretically either in the SG or near cell bodies
(Fleetward-Walker et al 1985a,b).

These results indicate that morphine-induced inhibition
occurs predominantly with administration in the SG, whereas
ENK-induced inhibition occurs both in the SG and near the
cell bodies of deeper neurones. Thus, it is possible that
morphine acts mainly presynaptically on the terminals of
primary afferents whereas enkephalins act postsynaptically on
neurones widely distributed in the spinal cord (Duggan,

It is puzzling that nearly all endogenous opioid peptides
do not have a high affinity for the μ-opioid receptor, the
site at which morphine is believed to act in producing analgesia (Kosterlitz 1985). Recently, metorphamide, a proenkephalin A derivative, was isolated from human adrenal medulla (Matsuo, Miyta, Mizuno 1983) and bovine caudate nucleus (Weber et al 1983). Bioassay and binding studies have shown that this amidated octapeptide has a high affinity for μ-receptors, negligible affinity for δ-receptors, while that for κ-receptors is about half that for μ-receptors (Weber et al 1983). Studies on the regional distributions of metorphamide demonstrated that this compound is located in the spinal cord of rat (Zamir et al 1985) and guinea pig (Sonders Barchas & Weber 1984). Functionally, intracerebroventricular metorphamide, an endogenous opioid peptide, has produced naloxone-reversible analgesia in mice and rabbits (Xu et al 1985). However, whether this peptide has analgesic effect at the spinal level is unknown although the reflex contractions of the rat urinary bladder were inhibited by intrathecal administration of metorphamide (Dray & Daris 1985). Experiments were therefore undertaken to determine whether this endogenous μ-preferring ligand has effects on nociceptive transmission in the spinal cord where morphine is known to have an action relevant to analgesia.

B. Segmental inhibition

Segmental control of sensory transmission in the spinal cord is a basic problem in neurophysiological studies. A segmental inhibition of group A primary afferent fibre activity on noxious input or C afferent-evoked responses of
dorsal horn neurones of the spinal cord has been well studied (Taub 1964 Mendell 1966, Hillman & Wall 1969, Wagman & price 1969, Brown, Kirk & Martin 1973, Wu, Chao & Wei 1974, Handerker, Iggo & Zimmermann 1975). On the basis of this inhibition, the gate control theory was proposed (Melzack & Wall 1965). I shall describe two types of segmental inhibition, "homoafferent nerve-" and "heteroafferent nerve-" induced inhibitions, depending on where conditioning stimuli are applied.

(a) Homoafferent nerve-induced inhibition (HMI)

This inhibition of nociceptive responses of dorsal horn neurones results from interaction between afferent volleys produced by conditioning and test stimuli which are applied to the same peripheral nerve in the cat (Wu, Chao & Wei 1974) and rat (Woolf & Fitzgerald 1982). For convenience, it is called "homoafferent nerve-induced inhibition" (HMI). Since it occurs in spinal preparations, this interaction is segmental. Based on this interaction, a practical technique, transcutaneous electrical nerve stimulation (TNS), was introduced to attempt to relieve certain types of experimental and clinical pain (Wall & Sweet 1967, Shao, Wu & Zhao 1978, review see Sjolund & Eriksson 1980). Similarly, in the traditional acupuncture practice, some special points located in a pain locus, called "Ah-Shi" points, are selected for stimulation in the control of pain (Beijing Traditional Chinese Medical College 1979).

The involvement of presynaptic inhibition in HMI was
first proposed by Melzack & Wall (1965). Support was provided by the finding that the conditioning volleys producing HMI increased the excibility of C fibre terminals in the rat spinal cord (Woolf & Fitzgerald 1982). Intracellular recording, however, indicated that segmental HMI was due at least in part to postsynaptic inhibition (Hongo, Jankowska & Lunberger 1968). Similarly, impulses in A-afferent fibres can inhibit L-glutamate-induced activity of dorsal horn neurones which is considered as an index of postsynaptic action (Besson et al 1974). In addition, since impulses relevant to both the testing and conditioning stimuli could be in the same fibers, the possible association of HMI with peripheral blockade was studied in human subjects (Campbell & Taub 1973, Ignelzi & Nyquist 1979). Moreover, conflicting evidence that there is no peripheral mechanism in HMI have been reported in the cat (Zhao, Yang & Shao 1978) and monkey (Lee et al 1985).

As mentioned above, opioid peptides and opiate receptors are highly concentrated in the region of termination of unmyelinated fibers and the nociceptive responses of dorsal horn neurones are inhibited by exogenous administration of opioid peptides (see section A). Whether opioid peptides are also involved in mediating segmental inhibition has been studied by administration of opioid antagonists. The results are controversial. TNS-induced analgesia in human subjects (Sjolund & Eriksson 1979) and HMI of dorsal horn neurones in the cat (Sinclair et al 1980, Lee et al 1985) were not reduced by systemic naloxone. However, the inhibition of C
evoked activity in lamina V neurones produced by A afferents in the rat (1-2 mg/kg) (Woolf & Fitzgerald 1982) and HMI on C reflex in the cat (0.25 mg/kg) (Cervero et al 1981) were reduced by i.v. naloxone. In behavioral experiments, systemic administration of naloxone partially reduced HMI in the monkey (0.05-0.1 mg/kg) (Huang et al 1978) and rat (1 mg/kg) (Woolf, Mitchell & Baret 1980).

(b) Heteroafferent nerve-induced inhibition (HTI)

This inhibition results from conditioning stimulation of afferent fibres other than those that evoke the test responses. When the conditioning stimuli excited only A afferent fibres, a short lasting inhibition (100-200 ms) of nociceptive responses occurred in dorsal horn neurones of the spinal cord (Wu, Chao & Wei 1974) whereas a very prolonged inhibition (more than 10 sec) of nociceptive responses occurred when unmyelinated afferents were excited by conditioning stimuli (Woolf 1983). Similarly, responses of the dorsal horn neurones to impulses in unmyelinated primary afferent fibres were inhibited by dorsal column stimulation which antidromically excites large myelinated afferent fibres (Foremen et al 1976, Lindblom, Tapper & Wiesenfeld 1977).

In contrast with peripheral nerve stimulation, DC stimulation involves in the excitation of homo- and hetero-afferent nerve fibres. Pharmacological studies showed no effect of naloxone on both HTI and DC-induced inhibition (Sinclair et al 1980).

It is known that there are high concentrations of GABA in the superficial dorsal horn of the rat (Patrick, McBride &
Felten 1979) and cat (Miyata & Otsuka 1975). Considerable evidence has indicated that GABA is an inhibitory transmitter in spinal cord and that spinal GABAergic neurones exert a presynaptic control of certain classes of primary afferent fibers (Curtis, Phillis & Watkins 1959, Curtis et al 1971, Game & Lodge 1975, Curtis, Lodge & Brand 1977, Curtis et al 1982, for review see Levy 1977). It has been found that GABA depolarizes the central terminals of large diameter cutaneous primary afferent fibers in the cat spinal cord (Gmelin & Zimmermann 1983) but evidence with C fibers is lacking. Recently, Duggan and Foong (1985) demonstrated that DC stimulation-induced inhibition of the excitation of dorsal horn neurones was reduced by microelectrophoretic administration of bicuculline, a GABA antagonist. The inhibition however was non-selective, reducing the responses of neurones to both noxious and non-noxious stimuli. As a consequence, it is possible that GABA is involved in HTI produced by A-afferents.

From the above mentioned investigations, although segmental inhibition has been studied extensively, its mechanism is still obscure. The investigations described in this part of thesis will focus mainly on the possible association of opioid peptides and GABA with Aδ afferents-induced segmental inhibition of nociceptive responses of dorsal horn neurones.

C, Tonic inhibition of motoneurone
Since the discovery of enkephalins, most attention has
been focused on their possible role in the control of transmission of nociceptive information. The findings that opioid antagonists increase spinal reflexes to noxious peripheral stimuli (Bell & Martin 1977, Jabb & Ramabadran 1978), and that stimulation of unmyelinated primary afferent fibres produces naloxone-reversible inhibition of a sural-induced gastrocnemius reflex (Catley, Clarke & Pascoe 1983, 1984), are consistent with this notion. But the curious finding that opioid antagonists increase monosynaptic reflexes produced by impulses in group I muscle afferent fibres, particularly of extensor muscles (Goldfarb & Hu 1976) is difficult to reconcile with such a role.

Earlier studies in both the spinal cat (Krivoy, Kroeger & Zimmermann 1973) and spinal dog (McClane & Martin 1967) found no effect on a variety of reflexes when naloxone was given in the dose range 0.2-1.0 mg/kg. Goldfarb & Hu (1976), however, found that naloxone 0.1-2.0 mg/kg increased monosynaptic reflexes in both flexor and extensor muscles and polysynaptic reflexes to impulses in large myelinated cutaneous afferent fibers in the spinal decerebrate cat. These investigators did not study reflexes to impulses in unmyelinated primary afferent fibre, but such reflexes were increased by naloxone (0.05 mg/kg) in the studies of Bell and his co-workers (1977, 1985). In the rabbit, it has been found that naloxone increased extensor reflexes of the hind limb more than flexor reflexes (Catley, Clarke & Pascoe 1983). In addition, low doses of naloxone also increased bladder reflexes (Roppolo, Booth & Groat 1983). For such a mechanism to enhance
monosynaptic reflexes, the inhibition must be tonically present since excitation from the peripheral stimulus is complete before any inhibition also produced by the same stimulus can have an effect. The functions of this tonic inhibition of motoneurones is unknown.

Although naloxone has been used extensively as a pharmacological tool in investigations of opioid peptides, the alteration of a physiological event by naloxone does not necessarily indicate the involvement of opioid peptides (Sawynok, Pinsky & La Bella 1979, Duggan & Johnson 1983). Since high doses of naloxone reduce inhibition by GABA (Dingleidine, Iversen & Breuker 1978, Gruol, Barker & Smith 1980), such doses might be expected to influence spinal reflexes. In the case of antagonism of the action of GABA, however, the effect of naloxone is not stereospecific, being produced by both (+)- and (-)-isomers (Gruol, Barker & Smith 1980). As a result, before an effect of an opioid antagonist can be related to opioid peptides, stereospecificity must be shown.

The following questions will be addressed in this thesis. Firstly, do opioid peptides antagonists elevate skeletal muscle reflexes to a variety of afferent inputs? Secondly, is this action stereospecific? Thirdly, does a similar inhibition operate to inhibit the transmission of this information to supraspinal regions? Experiments were also performed on whether opioid peptides are released near motoneurones or interneurones, so producing a tonic inhibition of spinal reflexes.
4.2. RESULTS

4.2.1. PHYSIOLOGY OF SPINAL INHIBITION

A. Segmental Inhibition

Inhibition of the nociceptive responses of dorsal horn neurones produced by electrical stimulation of large diameter primary afferent fibers can be divided into two sub-types. Homoafferent nerve induced-inhibition (HMI) results from conditioning stimuli applied to the nerve which also contain the fibers producing the test responses. Thus, this is really a period of inhibition which follows an evoked response. As explained in methods, the conditioning stimuli ceased just prior to the evoked response. Heteroafferent nerve induced-inhibition (HTI) results from conditioning stimuli in primary afferents of the nerve other than those which produce the test responses. The conditioning stimulus was applied throughout the evoked responses.

(a) Homoafferent Nerve Induced-Inhibition (HMI)

The responses of 46 neurones in laminae I, III-V of the dorsal horn were recorded extracellularly. Except for 5 nociceptive specific neurones, the remaining 41 were WDR neurones excited by both noxious and innocuous cutaneous stimulation.

When using noxious heating of the hind limb digits as a test stimulus, conditioning electrical stimuli were applied to the ipsilateral tibial nerve. The threshold of the tibial
nerve was measured by recording a volley in a dorsal root near the spinal cord. Since plantar nerves are branches of the tibial nerve and innervate the glabrous skin of the plantar region where the noxious heat was focussed, electrical stimulation of the ipsilateral tibial nerve as a conditioning stimulus would include fibers innervating the area of skin used to excite each neurone. This homoafferent conditioning stimuli (HMS) always evoked repetitive firing of nociceptive neurones at the beginning of stimulation but this firing declined rapidly with continued stimulation. When the intensity of a conditioning stimulus exceeded 5 T, the activity of neurones (32/34) produced by natural stimuli (hair deflection and noxious heating), and the firing of neurones (11/12) by electrical stimulation of A and C fibres of the tibial nerve, was non-selectively reduced. By increasing the intensity of the conditioning stimulation to 20 T, the firing of 10 of 18 neurones produced by noxious and non-noxious stimuli was almost completely abolished, that of the remaining 8 neurones was also markedly inhibited. An example is illustrated in Fig.12.

(i) Failure of Unmyelinated Afferent Blockade in HMI

Since the afferent impulses from the test noxious stimuli and those from the conditioning stimuli travelled to the spinal cord through the same nerve, a peripheral mechanism of inhibition of responses to the test stimuli needed to be considered.

In 4 experiments, the multiunit afferent discharges
Fig. 12. The inhibition of dorsal horn neurones produced by homoafferent nerve stimulation (HMS). A, Non-selective reduction of noxious heat responses and hair deflection responses of a lamina IV neurone by HMS (0.2ms pulses, 100Hz, 20T for 20sec). B, The recordings show the number of action potentials of a lamina V neurone evoked by impulses in C fibre primary afferents following electrical stimulation of the ipsilateral tibial nerve at 0.6 Hz and inhibition of the gated C responses by HMS (20T).
recorded from 6 filaments of the left plantar nerve at a proximal site, and the firing of 6 dorsal horn neurones, were recorded simultaneously during noxious heat stimulation of the glabrous skin of the plantar region. When a conditioning stimulus (0.2ms pulses, 100Hz, 20T for 20sec) was applied to a distal site of the plantar nerve, multiunit discharges recorded in 5 plantar filaments to noxious heating were unchanged until the conditioning stimulus was 100-200 T at which strength unmyelinated afferent fibres were excited. At these high stimulus intensities, antidromically conducted impulses from the conditioning stimuli would collide with some orthodromically conducted impulses from the cutaneous heating, so that the latter would not be recorded from a plantar nerve. By contrast, the firing by noxious heat of 4 of 5 dorsal horn neurones was potently inhibited at conditioning stimuli of 20 T. One was not inhibited. A slight depression of plantar afferent discharges in one filament was produced by conditioning stimulation at 20 T. Unfortunately a neurone excited by this plantar afferent volleys was not found in the dorsal horn. As shown in Fig.13 conditioning stimulation at 20 T did not alter the plantar afferent discharges whereas an intensity of 10 T completely inhibited nociceptive heat responses in a neurone of the dorsal horn, indicating that peripheral blockade of impulses is not important in the observed HMI.

(ii) Effect of Naloxone on HMI

Of 15 neurones in laminae IV-V, HMI of 13 WDR
Fig. 13. Lack of unmyelinated afferent blockade in homoafferent nerve-induced inhibition (HMI). A, No effect of homoafferent nerve stimulation (HMS) (plantar stimulation at 20T) on multiunit discharges recorded in a filament of the plantar nerve to noxious heating (50°C). B, Almost complete inhibition of noxious heat response in a lamina V dorsal horn neurone by HMS (20T).
neurones was partially reduced by the opioid antagonist naloxone when microelectrophoretically administered in the SG (70-150 nA, mean 121 nA, 2-18 min, in 7 neurones) or near cell bodies (30-80 nA, mean 60 nA, 5-14 min, in 6 neurones). Fig.14 illustrates results from one neurone and fig.15 summarizes the mean reduction by naloxone of HMI of 13 neurones. As shown in Fig.15, prior to naloxone administration, conditioning stimuli produced reductions of noxious heat responses to 80.1% ± 6.7 S.E.M. (n=13) of control. After ejection of naloxone, noxious responses were reduced to 63.5% ± 7.1 S.E.M. (n=13) of control. With the remaining two nociceptive-specific neurones (responding only to noxious heat) in lamina IV and all of 6 neurones in lamina I, the HMI was unaltered by naloxone regardless of whether this compound was administered in the SG or in the vicinity of cell bodies.

(iii) Failure of bicuculline to reduce HMI

In contrast to the effect of naloxone, microelectrophoretic administration of bicuculline methochloride, a GABA antagonist, in the SG or near cell bodies produced no apparent effect on the HMI with 11 of the 13 neurones studied. The HMI in the remaining 2 neurones was slightly reduced.

(b) Heteroafferent Nerve Induced-Inhibition (HTI)

Twenty-five neurones were recorded at depths of 1.07-2.1 mm in the dorsal horn of spinal cord. They were located
Fig. 14. Reduction of homoafferent nerve-induced inhibition (HMI) by naloxone. The excitation of a lamina IV dorsal horn neurone to noxious heat (50°) on the skin of hindlimb digital pad and deflection of adjacent hairs was inhibited by homoafferent nerve stimuli (HMS) at 20 T. Microelectrophoretic administration of naloxone in the SG (150 nA, 7 min) partially reversed this HMI.

Fig. 15. Partial reversal of homo-afferent nerve-induced inhibition (HMI) following microelectrophoretic naloxone. The histograms show the inhibition by HMS of nociceptive responses calculated as a percentage of control (n=13). The bars indicate standard errors of the means.
Fig. 14

![Graph](image)

**Graph Legend:**
- NOXIOUS HEAT
- HAIR DEFLECTION
- HMS: HOMOAFFERENT NERVE STIMULATION

---

Fig. 15

![Bar Graph](image)

**Bar Graph Legend:**
- BEFORE NALOXONE
- AFTER NALOXONE

**N=13**
in laminae I (2 cells), III (5 Cells), IV (8 cells) and V (10 cells). Among them, 20 neurones responded to noxious heating and hair deflection. The remaining 5 were excited by electrical stimulation of the peripheral nerve. The contralateral tibial nerve was electrically stimulated as the heteroafferent nerve conditioning stimulus. Of 25 neurones, the firing of only 3 neurones (2 excited by C volleys, 1 by noxious heat) was depressed by conditioning stimulation at 10T. With the increase of conditioning stimulation to 20T, of 17 neurones tested, 47.1% (1 excited by C volleys, 7 by noxious heat) were inhibited. When conditioning stimulation was given at 30T, 80% (4/5) of neurones responding to noxious heat were inhibited. As shown in Fig.16, heteroafferent nerve conditioning stimulation at 20T partially inhibited the firing of a neurone produced by noxious heating (50°C). Fig.17 illustrates that A- and C- responses of a neurone elicited by stimulation of the ipsilateral tibial nerve were non-selectively inhibited. With continuous conditioning stimulation (50Hz), inhibition was not sustained but declined such that control responses were observed within 2 minutes of the start of peripheral nerve stimulation.

(i) **Effect of Bicuculline on HTI**

Of 11 neurones tested, the HTI of 7 neurones was partially reduced by microelectrophoretic administration of bicuculline. When bicuculline (90-150 nA for 4-18 min) was ejected in the SG, the HTI of 5 of 6 tested neurones was
Fig. 16. Inhibition of a dorsal horn neurone produced by heteroafferent nerve stimulation (HTS). The firing of a lamina IV neurone produced by noxious heating (50°C) and hair deflection. HTS (0.2ms pulses, 50Hz, 20T for 20sec) inhibited noxious heat response.

Fig. 17. The post-stimulus histograms represent 16 summed responses to electrical stimulation of the ipsilateral tibial nerve with a stimulus strength more than 50T. The spikes appearing at latencies 10-20ms and 150-200ms were produced by impulses in myelinated and unmyelinated primary afferent fibres, respectively. Note that A- and C-responses were inhibited by HTS (repetitive 0.2ms pulses, 50Hz, 20T).
reduced. An example was shown in Fig.18, which illustrates the ejection of bicuculline (90 nA) in the SG and a reduction in the HTI of C responses of one neurone. When administered near cell bodies (50-90 nA for 6-30 min), the inhibition was reduced with 3 of 5 neurones. Fig.19 illustrates that the reduction by bicuculline (administered near the cell body) of the HTI of noxious heating responses of one neurone was reversible.

(ii) Failure of Naloxone to influence HTI

The HTI of nociceptive responses of dorsal horn neurones was not blocked when naloxone was administered microelectrophoretically in either the SG (80-100 nA for 17-25 min, 2 cells) or the vicinity of cell bodies (150 nA for 11-15 min, 2 cells). It is of interest that with one neuron, when a strong conditioning stimulation (10 V) was applied unexpectedly, gated C responses were reduced to 46% ± 0.04 S.E.M. (n=20) of control, and then during the ejection of naloxone (80 nA for 10 min) C responses were reduced to 34.2% ± 0.68 SEM (n=20) of control, suggesting a slight blockade of the HTI resulting from the large stimulus.

B. Tonic Inhibition of Motoneurones

(a) Non-selective facilitation by opioid antagonists of spinal reflexes to stimulation of myelinated and unmyelinated primary afferent fibres

Reflexes recorded in ventral roots were produced by
Fig. 18. Effect of bicuculline on heteroafferent nerve-induced inhibition (HTI) of gated C responses of a lamina V dorsal horn neurone to electrical stimulation of unmyelinated primary afferent fibres. The gated C response was plotted continuously and the graph plots the means of 20 such responses with respect to time. Note that reduction of HTI after microelectrophoretic administration of naloxone in the substantia gelatinosa (SG) (90nA for 16min). The bars indicated standard errors of the means.
Fig. 19. Effect of bicuculline on heteroafferent nerve-induced inhibition (HTI) of a dorsal horn neurone. The recording shows the total number of action potentials evoked by noxious heat (50°C), determined with a microprocessor-controlled integrator which sampled and subtracted spontaneous firing. Note the partial reversal of HTI produced by microelectrophoretic administration of bicuculline (80 nA) near the cell body.
stimulation of the nerves to biceps semitendinosus (BST), the medial and lateral gastrocnemius (ML-G), the sural and the tibial nerves in 10 experiments. Usually, stimuli of less than 10 T were used to elicit reflexes, and thus it is improbable that unmyelinated afferent fibres were excited.

(i) Monosynaptic reflexes to impulses in group I afferent fibres of the BST or ML-G nerves

The latencies of BST and ML-G reflexes of durations less than 1.2 ms were within the range 3.0–3.4 ms and 3.5–4.0 ms, respectively. Intravenous naloxone (0.025–0.1 mg/kg) significantly enhanced both the BST and ML-G monosynaptic reflexes in all experiments (Table IV and Fig. 20 A). Since (+) naloxone was not available, the (+) and (-) isomers of furylmethylnormetazocine (FMN) were used to assess the stereospecificity of this effect. Only the (-) isomer is an opiate antagonist (Kuhn & Stockhaus 1976). Increases in the BST and the ML-G monosynaptic reflexes were also produced by intravenous (-)-FMN in doses of 0.03–0.2 mg/kg (Fig. 20 B). The mean increases are shown in Table IV.

<table>
<thead>
<tr>
<th>REFLEX</th>
<th>NALOXONE (0.05mg/kg)</th>
<th>(-)-FMN (0.03mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>BST</td>
<td>80.9±22.3(n=9)</td>
<td>27±8.8(n=4)</td>
</tr>
<tr>
<td>ML-G</td>
<td>138.2±67.9(n=8)</td>
<td>65±40.3(n=3)</td>
</tr>
<tr>
<td>SURAL</td>
<td>26.6±12.9(n=8)</td>
<td>32.8±10.1(n=4)</td>
</tr>
<tr>
<td>TIBIAL</td>
<td>43.3±8.3(n=8)</td>
<td>48.5±13.9(n=4)</td>
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</table>
(+)-FMN in the same dose range as (-)-FMN did not increase BST monosynaptic reflexes in all 4 experiments, had no effect on the ML-G monosynaptic reflexes in 2 experiments and decreased them in one. It should be noted that although the amplitude of the BST monosynaptic reflex increased with both (-)-opioid antagonists, naloxone and (-)-FMN, no new peaks of later firing were recorded. Since the first peak of firing was complete within 1.2 ms, the increased amplitude must have resulted from recruitment of previously silent motoneurones and not from repetitive firing of active motoneurones.

(ii) Reflexes to stimulation of large myelinated afferent fibres of the sural or tibial nerve

Compared with the monosynaptic reflex to group I muscle afferents, these reflexes had longer latencies (4.0-6.6 ms) and were broadly dispersed when stimulating below 4T. As shown in Table IV, naloxone enhanced reflexes to sural stimulation in 8 experiments (Fig. 20 A).

(-)-FMN increased the sural and tibial reflexes in all 4 experiments (Table IV & Fig. 20 B). (+)-FMN slightly increased the sural and tibial reflexes in 2 cases, having no effect in 2. The increase of sural and tibial reflexes produced by (+)-FMN was small when compared with the effect of subsequent (-)-FMN at the same dose in one experiment and in the other both the (+) and (-) isomer produced similar small increases.
Fig. 20. Enhanced spinal reflexes following intravenous naloxone and (-)- but not (+)-FMN. Each pen recording is the average 16 Sl ventral root reflexes produced by electrical stimulation of a peripheral nerve.

A, Naloxone and reflexes evoked by BST and sural nerve stimulation. First controls, 2 stimuli of 3T and 4T to the BST nerve, and 2 stimuli of 7T and 10T to the sural nerve; Second controls, 2 stimuli of 2T and 5T to the BST nerve, and 2 Stimuli of 8T and 10T to the sural nerve. Note that following the first dose of naloxone (0.05mg/kg) 3 h were allowed for elimination of the drug and then new controls using different stimulus parameters were obtained. A second dose of 0.05mg/kg of naloxone produced small increases in reflexes and those illustrated are following a larger dose (0.5mg/kg). The increases produced by naloxone were in both cases recorded 9-12 min after the antagonist was given intravenously.

B, (+)- and (-)-FMN and reflexes evoked by BST and sural nerves stimulation. BST nerve stimulation: 1 stimulus of 6T. Sural nerve stimulation: 1 stimulus of 3T. The records illustrated for (-)- and (+)-FMN (0.3mg/kg) were obtained within 5-8 min of administering each drug.
(iii) Reflexes to impulses in C primary afferent fibres of the tibial nerve

Naloxone (0.025-0.05 mg/kg) increased reflexes to stimulation of C fibre afferents of the tibial nerve in all 9 cats. The mean increase was 138.6% ± 49.2 S.E.M. (n=9).

(b) A Contrast in the Effects of Naloxone on Transmission of Primary Afferent Information to Ventral Roots and to Ascending Tracts of the Spinal Cord

(i) Impulses in unmyelinated primary afferent fibres

In 12 experiments, naloxone in a dose of 0.05 mg/kg i.v. increased the C response recorded in the contralateral anterolateral fasciculus (ALF) from electrical stimulation of unmyelinated primary afferents of the tibial nerve. The mean increase above controls produced by this dose of naloxone was 60.9% ± 9.9 S.E.M., calculated for observations made in the period 3-8 min after the administration of naloxone.

In 5 experiments, the ventral root reflexes and ALF volleys to impulses in unmyelinated primary afferent fibres were measured alternately. Both reflexes and ALF volleys were increased in all 5, but the increase in the reflex was greater than the increase in ascending volleys. Fig. 21 shows results from one experiment. Naloxone was given in two doses of 0.05 mg/kg. There is clearly a dose dependency in the effect of naloxone on the ventral root reflex, but this is not present in the increase of ascending impulses.
(ii) Impulses in group I muscle afferents

Since monosynaptically relayed ascending impulses from group I muscle afferents travel through the dorsal spinocerebellar tract (DSCT), the DSCT volley was recorded from the region of the dorsolateral fasciculus (DLF). A DSCT volley of latency 3.2-5.6 ms (the longer latencies were at C2) followed electrical stimulation of group I afferent fibres of the combined ML-G nerves. In all 5 experiments naloxone (0.05 mg/kg) did not significantly increase the amplitude of the DSCT volley.

Monosynaptic reflexes in the ventral roots were recorded in 4 of these tests. The increases (calculated from the areas of the averaged records) following by naloxone were 25, 128, 148 and 240%. Fig.22 shows this vast difference in the effects of naloxone on monosynaptic relay of group I muscle afferents into the ventral roots and into the ipsilateral DLF.

(iii) Impulses in large diameter cutaneous afferent fibres

These were studied in two ways. In 3 tests the sural nerve was stimulated with a strength of up to 6 T and an ascending compound action potential recorded in the contralateral ALF. In addition, in 4 experiments in which volleys in the ALF were monitored by gating for impulses related to unmyelinated primary afferent fibres of the tibial nerve, histograms were concurrently compiled and these
Fig. 21. Effects of i.v. naloxone on ventral root reflexes and ascending impulses in the anterolateral spinal fasciculus (ALF) to electrical stimulation of primary afferent C fibres of the tibial nerve. The numbers of impulses evoked by stimulation of the tibial nerve (200 T) adequate to excite C fibres are plotted with respect to time. The counts relate only to impulses produced by stimulation of C fibres (see Methods). A, Recordings in the ipsilateral S1 ventral root. The mean C reflex response in the control period was 30.3 ± 0.9 S.E.M. (n=16) spikes. After naloxone 0.05 mg/kg it rose to 96.6 ± 4.2 and after the second dose of 0.05 mg/kg the mean was 183.4 ± 4.7. B, Recordings from the contralateral ALF. The one counting system was used alternately for the two signal sources. The first dose of naloxone (0.05 mg/kg) was given at time 0. The second (0.05 mg/kg) 7 min after the first. The control C response was 130.4 ± 3.6 S.E.M. (n=16) spikes and after the dose of naloxone rose to 201.3 ± 2.8; after the second dose, the mean was 210.3 ± 2.8.

Fig. 22. Naloxone and group I muscle afferents. A, Reflexes recorded in the ipsilateral ventral root to electrical stimulation of the ML-G nerves with a strength of 16 T. Each record is the average of 16 responses. Both the ventral root reflex and the ascending volley were detected with a stimulus of 2 T but the larger stimulus gave more consistent records. B, Recording from the ipsilateral DLF at the T9 segment. Each record is the average of 32 responses. Naloxone was given 4 min before the right hand record in A and 5.5 min before that in B. The nerve stimuli were delivered at times 0.
Fig. 21

A

VR REFLEX

NALOXONE 0.05mg/kg

NALOXONE 0.05mg/kg

B

ALF VOLLEY

NALOXONE 0.05mg/kg

Fig. 22

A

CONTROLs

NALOXONE 0.05mg/kg

VR REFLEX

B

DLF VOLLEY

200µV

50µV

0  15 MS

0  15 MS
contained an early peak produced by impulses in large
diameter primary afferent fibres of that nerve. The latter
are mainly of cutaneous origin but would also include group I
afferent fibres of the intrinsic muscles of the hind paw.
Thus these results cannot be related to one fibre grouping
but they are still useful for contrasting the effects of
naloxone on transmission of impulses in large diameter
afferents with effects on impulses in unmyelinated primary
afferent fibres.

When the sural nerve was stimulated, the ascending volley
was increased by 35% in one experiment after i.v. naloxone
(0.05 mg/kg) (ventral root not measured) and was unchanged in
two (ventral root reflexes increased by 16 and 75%). In one
of these experiments (shown in Fig.23), with a further dose of
naloxone (0.05 mg/kg) the ascending volley was unchanged but
the ventral root reflex increased to 150% above controls.

With stimulation of the tibial nerve, no significant
increase in the early peak of the potentials recorded in the
ALF occurred in all 4 experiments following naloxone (0.05
mg/kg). Thus of a total of 7 experiments, in only one was
the ALF potential produced by impulses in large diameter
primary afferent fibres increased following naloxone.

These results show that i.v. naloxone increased ventral
root reflexes in response to stimulation of all primary
afferent fibre types but of the ascending volleys, only those
to stimulation of unmyelinated primary afferent fibres were
increased.
Fig. 23. Naloxone and large diameter cutaneous afferents.

A, Reflexes recorded in the ipsilateral S1 ventral root to electrical stimulation of the sural nerve with a strength 2.5 X threshold.  B, Volleys recorded in the contralateral ALF at L1 to the same stimuli as in A.  All records are the average of 16 responses. The nerve stimuli were delivered at time 0. The middle records were obtained between 5 and 6 min after administration of naloxone (0.05 mg/kg) and the right hand records between 5 and 6 min after a second dose of 0.05 mg/kg. The first and second doses of naloxone were separated by 9 min.
(c) Naloxone and the excitatory postsynaptic potentials (EPSPs) of motoneurones produced by stimulation of peripheral nerves

(i) Increase of EPSPs by systemic administration of naloxone

Intravenous naloxone (0.05-0.10 mg/kg) increased the amplitude of EPSPs in 10 of 16 motoneurones. In 6 of 10 cases, these increases in EPSPs occurred without changes in membrane potential. An example is shown in Fig. 24, indicating the nonselective action of naloxone on EPSPs of a motoneurone evoked by different nerve stimuli. Prior to naloxone the neurone did not fire to any nerve stimulus but after a total dose of 0.1 mg/kg it fired constantly to an ML-G stimulus, irregularly to the sural stimulus, and EPSPs from BST and tibial stimulation were increased.

Somatic membrane conductance was measured in 10 neurones. With 9, the membrane conductance was not altered by naloxone. In one neuron a slight decrease in conductance was found. As shown in Fig. 24b, the current/voltage relationships after naloxone from a motoneuron were similar to controls. No significant alteration in antidromic action potential amplitude was produced by naloxone (Fig. 24c).

(ii) Failure to increase EPSPs by microelectrophoretic administration of naloxone near motoneurones

Using parallel micropipette assemblies, intracellular recordings were obtained from 20 motoneurones.
Fig. 24. Increased EPSPs but no change in somatic membrane conductance of a motoneurone following i.v. naloxone.

A, EPSPs evoked by stimulation of the ML-G nerve (1.6 T), BST nerve (1.8 T), tibial nerve (1.5 T) and sural nerve (1.5 T). Each record is the average of 16 EPSPs evoked at 1 Hz. A 2mV calibration pulse is present on each record and the trailing edge of the stimulus pulse coincides with the trailing edge of the calibration. Amplifier time constant, 1 second.

After naloxone 0.10 mg/kg an action potential was evoked constantly by ML-G stimulation but irregularly by sural stimulation. Since averaging the latter gave a misleading record, 1 sweep only is illustrated in the post-naloxone record. Voltage/current relationships are plotted in B for the same neurone both before and after i.v. naloxone. The current pulses were 20 ms in duration. The antidromic action potential before and after naloxone is show in C.
The data presented is derived from recording electrical potentials greater than 10mV and NC which are depicted by red. The in vitro experiments where neuronal activity from isolated preparations was measured. This type of result indicates the presence or absence of selected compounds from the region around the tip of the electrode. A partial separation of the tip of the electrode was noted, which indicated the presence of a compound. A comparison of the present data with previously reported studies is noted. The control responses were reproducible and validated through additional experimental setups. A summary of the results and discussion is presented.
The data presented is restricted to motoneurones with resting membrane potentials greater than -50 mV and shown to be depolarized by DLH. The latter is important since failure of a compound to affect the membrane potential in experiments of this type can result from tissue debris preventing free diffusion of ejected compounds from the region around the tip of the micropipette, partial separation of the tip of the drug administering pipette from the recording electrode as well as inactivity of the compound being tested.

**Naloxone** Twenty neurones were depolarized by DLH ejected with currents of 50-150 nA for 1-3 min. The depolarization typically required one minute to reach a maximum and varied from 3 to 11 mV (mean 7.4 mV + 0.46 S.E.M.). Examples are shown in Fig.25 and 26. On 14 of these neurones, naloxone (40-200 nA for 3-14 min), had no effect on resting membrane potential and failed to increase EPSP amplitudes with any neurone. With 3 motoneurones EPSP amplitudes were decreased during naloxone ejection. Fig.25 shows the failure of naloxone (100 nA) to increase the EPSP amplitude a motoneurone depolarized by DLH.

Effects by glycine (5-100 nA) were observed less frequently than with DLH, and hyperpolarization was observed consistently with only 6 of 16 neurones. An example is shown in Fig.25. On 4 of these neurones naloxone (40-125 nA) did not alter resting membrane potential. Naloxone also failed to increase EPSP amplitude, there being no change with 3 neurones and a small decrease with one.
Fig. 25. Lack of effect of met⁵-enkephalinamide (MENKA) on resting membrane potential and lack of effect of naloxone on an EPSP of a motoneurone. A, A pen recording of resting membrane potential. The times of ejection of DL-homocysteate (DLH, 40 nA), glycine (5 nA) and MENKA (60 nA) are shown by continuous bars. Note the depolarization by DLH, hyperpolarization by glycine, but lack of effect of MENKA. B, Pen recordings of averaged monosynaptic EPSPs. Each record is the average of 16 EPSPs evoked by electrical stimulation of the PBST nerve at 2 T at a frequency of 1 Hz before, during naloxone ejection, 100 nA for 10 min, and after. Each EPSP is preceded by a 2mV calibration pulse and the nerve stimulus coincides with the trailing edge of this pulse.
Opioid peptides Both Met$^5$-enkephalinamide (MENKA) and D-Ala$^2$-Leu$^5$-enkephalin (DADL) were tested on motoneurones shown to be depolarized by extracellular administration of DLH. Ejected with currents 60-100nA for 3-12 min, MENKA had no effect on the resting membrane potential of all eight motoneurones tested. These opioid peptides also had little effect on motoneurone EPSPs (5 cells tested with MENKA, 3 with DADL). On only one motoneuron was a decrease in the excitatory postsynaptic potential (EPSP) amplitude observed, and recording conditions deteriorated before antagonism by naloxone could be tested.

Unlike the opioid peptides, EPSPs were reduced by glycine. EPSPs were recorded before, during and after glycine ejection (80-100 nA) with 5 motoneurones, only one of which was hyperpolarized by the currents used. With 3 of 4 motoneurones, despite a lack of effect on recorded membrane potential, glycine produced relatively large decreases in EPSP amplitude. Results from one neurone are shown in Fig.26. EPSPs were also decreased with the remaining neuron which was also hyperpolarized by glycine. Opioid peptides were tested on all of these 4 neurones, but unlike glycine no decrease in EPSP amplitudes were detected (MENKA 3 cells, DADL 1 cell). On these same neurones, in addition to reducing the amplitude, glycine also slowed the rate of rise of EPSPs. This parameter, however, was unchanged by ejection of MENKA and DADL.
Fig. 26. Reduction of a motoneurone EPSP by glycine but not by met5-enkephalinamide (MENKA). A. The upper record is a pen recording of resting membrane potential of a motoneurone. Note the depolarization by DLH but lack of effect by glycine. This current of glycine however greatly reduced the amplitude of the EPSP evoked by stimulation of the ML-G nerves at 1.5 T, 1 Hz. The EPSP records are averages of 16 responses. B, MENKA had no effect on membrane potential or the EPSP. There is a resistive coupling artefact present with all three compounds on the membrane potential records.
4.2.2. PHARMACOLOGY OF SPINAL INHIBITION

A. Selective inhibition by metorphamide, a high affinity endogeneous µ ligand, of dorsal horn neurones

As outlined in the introduction (4.1), morphine, predominantly a µ ligand, suppresses the transmission of impulses in nociceptive afferent fibres when administered in the SG, but has minimal effects on lamina IV/V neurones when administered near the bodies of these cells. Thus when examining the actions of metorphamide, this peptide was administered from micropipettes both in the SG and near the bodies of deeper neurones.

The effects of metorphamide were studied on 18 dorsal horn neurones. One neurone was in lamina I, 9 were in lamina IV and 8 in lamina V, estimated by depth from the dorsal cord surface. All but one were WDR neurones, responding to noxious and innocuous cutaneous stimuli. The remaining one (in lamina V) appeared to be nociceptive-specific since it responded to noxious but not innocuous cutaneous stimuli.

(a) Metorphamide Administered in the Region of the Substantia Gelatinosa

The effects of 14 administrations of metorphamide in the SG were studied with 10 neurones. With 8 neurones, responses to alternate noxious heating and hair deflection were studied and with 2, gated C responses were recorded. With all 10 neurones, metorphamide ejection (50-150 nA, mean
75 nA, for 5-17 min) in the SG reduced noxious heating and C responses, to a mean of 42% ± 7 S.E.M. (n=14) of control responses (Fig.27,28). This inhibition was selective for nociceptive responses since excitation by hair deflection was reduced with only one of 3 neurones (Fig.28A). Spontaneous firing, however, was reduced by metorphamide with 6 of 9 administrations, to a mean of 36% ± 11 S.E.M. (n=6) of controls. Upon cessation of metorphamide ejection, the recovery time for nociceptive responses ranged from 4 to 9.5 (mean 7) minutes.

To investigate whether these effects were due to an action at opioid receptors, naloxone was electrophoretically administered at the same SG site with 9 of the 10 neurones tested. With 9 neurones, naloxone (55-150 nA, mean 130 nA) was administered with metorphamide, commencing at the time of maximal depression of nociceptive responses (Fig.27,28). Recovery commenced within 3-9 (mean 4.6) min after starting naloxone administration, with nociceptive responses eventually reaching a mean of 101% ± 12 S.E.M. (n=7) of control values (Fig.27,28). In only 2 cases, however, did the nociceptive response clearly exceed control levels, the "overshoot" phenomenon described with opiates such as morphine Duggan, Hall & Headley 1977). With the remaining 2 neurones, naloxone administration preceded metorphamide administration and blocked the reduction of nociceptive responses by metorphamide. With 2 neurones, met\textsuperscript{5}-enkephalinamide was administered at the same SG site as metorphamide, producing selective inhibition of nociceptive
responses, confirming previous observation (Duggan, Johnson & Morton 1981).

(b) **Metorphamide administered near the cell bodies of dorsal horn neurones**

The effects of 13 administrations of metorphamide near cell bodies were studied with 10 dorsal horn neurones. With 6 of these neurones, the proximity of the tip of the seven-barrel micropipette to the cell bodies was confirmed by excitatory or inhibitory effects induced by ejection of DLH or glycine, respectively. With all 10 neurones, metorphamide administration (30-50 nA, mean 43 nA, for 3-11 min) reduced nociceptive heat responses, to a mean of 27% ± 4 S.E.M. (n=13) of control responses (Fig.28B). After cessation of metorphamide ejection, noxious heat responses recovered in 4-45 min (mean 15 min). For 9 administrations this inhibition was selective since responses to hair deflection were not concomitantly reduced. With the remaining 7 administrations, however, these responses were also decreased, to a mean of 69% ± 5 S.E.M. (n=7) of controls. Spontaneous firing was reduced with 12 of 13 ejections of metorphamide, to a mean of 33% ± 6 S.E.M. (n=12) of controls. Thus, overall, metorphamide ejected near cell bodies produced a relatively greater reduction of responses to noxious compared with non-noxious skin stimuli, but was less selective when compared with SG administration. With 6 neurones naloxone (20-85 nA, mean 53 nA) was administered microelectrophoretically during inhibition by metorphamide and at the same site (Fig.28B).
Fig. 27. Reduction of gated C responses of a lamina IV dorsal horn neurone by microelectrophoretic administration of metorphamide in the substantia gelatinosa (SG), and reversal of this effect by concomitant microelectrophoretic administration of naloxone. Each plotted point is the mean (n=4) number of action potentials evoked by electrical stimulation of C primary afferent fibres of the ipsilateral tibial nerve (0.3 Hz). Standard error bars are indicated.
Fig. 28. Effect of microelectrophoretic metorphamid on the nociceptive excitation (heating of a digital pad) and non-nociceptive excitation (deflection of adjacent hairs) of dorsal horn neurones. A, Selective reduction of noxious heat responses of a lamina IV neurone by metorphamide administered in the SG. Subsequent administration of naloxone in the SG reversed this depression. B, Relatively selective reduction of noxious heat responses of a lamina IV neurone by metorphamide administered near the cell body. Response to hair deflection were partially reduced. Microelectrophoretic administration of naloxone at this site reversed this effect.
With all 6 neurones, naloxone reversed the opioid-induced inhibition, commencing within 2-5 min (Fig. 28B). Of the 4 neurones tested until full recovery, noxious heat responses exceeded control values with 3 neurones. Morphine and metorphamid were administered microelectrophoretically near cell bodies of the same 4 neurones. With all 4 neurones, morphine (30-70 nA) had no effect or produced excitation with firing in bursts whereas metorphamid was inhibitory.

B. Effects of peptide inhibitors on opioid peptide-induced inhibition of dorsal horn neurones

Both D-phenylalanine and kelatorphan were examined for possible potentiation of the action of exogenously administered met^{5}-enkephalin (MENK), and subsequently for modification of synaptic events in the spinal cord. In particular, physiological events known to be altered by naloxone were studied.

(a) D-phenylalanine (DPA)

Results were obtained from 47 dorsal horn neurones. On the basis of depth from the dorsal surface of the spinal cord, 2 were in lamina I, 23 in laminae III and IV and 22 in lamina V. For test purpose, 26 neurones were excited synaptically by noxious and non-noxious cutaneous stimulation while others were excited by continuous DLH ejection (10-40 nA).

(i) Non-selective depression of cell firing by DPA per se

DLH-induced firing : Since spontaneous and evoked
firing may be generated at synapses widely distributed on neurones, this type of firing may be relatively difficult to inhibit with a compound administered from a electrode tip positioned near a cell body. Firing by an excitant also administered from the electrode tip is more readily inhibited since both the excitant and the depressant reach approximately similar regions of each neurone. Of 13 neurones excited continuously by the ejection of DLH, 12 were inhibited by DPA ejected with relative low currents (5-40 nA, mean 13). Fig.29B illustrates dose-dependent depression by DPA of DLH-induced firing of a neurone in lamina V. This figure also shows that the ejecting currents of DPA needed to depress cell firing were far greater than those of glycine.

**Firing by noxious heat:** When ejected near spinal neurones DPA (30-100 nA, mean 70 NA) was a non-selective depressant of cell firing. Thus with all 4 WDR neurones in which firing to noxious and non-noxious cutaneous stimuli were examined concomitantly, both responses were reduced similarly together with spontaneous firing. With a further 4 WDR neurones only excitation by noxious heat was examined quantitatively, and this was reduced in 3. DPA also reduced excitation by hair deflection with all 3 neurones not shown to be excited by noxious heating of the skin. Fig.29A illustrates non-selective depression of excitation of a lamina V neurone by noxious and non-noxious cutaneous stimulation. Recovery from the currents of DPA (80 nA) needed to depress synaptic responses occurred within 3-4 min. DPA was also administered microelectrophoretically in the SG
while recording the responses of deeper neurones. Using currents greater than those used near cell bodies (80-100 nA) no change in synaptic responses was observed with all 6 neurones studied.

Intravenous DPA (20-70 mg/kg) depressed the nociceptive and non-nociceptive responses of 3 of 6 WDR neurones. With 3 neurones, spontaneous firing was reduced. Fig.29C shows these actions of i.v. DPA graphically.

Effects of naloxone on DPA-induced depression: Since analgesia from DPA administration to mice was found to be reversed or prevented by naloxone (Ehrenpreis et al 1979), this opioid antagonist was given both electrophoretically and intravenously. Administered microelectrophoretically near 3 neurones, naloxone partly reversed the depression of nociceptive responses by similarly administered DPA. With these neurones, however, naloxone alone increased spontaneous firing. In 2 experiments, depression of synaptic responses by intravenous DPA (35mg/kg) was reduced by intravenous naloxone (0.1 mg/kg). Although the effect of naloxone alone was not examined in these experiments, spontaneous firing was not elevated by the doses of naloxone capable of reducing the depression of synaptic responses by DPA.

(ii) Studies on the action of exogenous MENK by DPA

The depressant action of DPA per se could occur by potentiation of tonically released MENK or other actions. To examine this, the interaction between MENK and DPA was studied.
Fig. 29. Depression of neuronal firing by D-phenylalanine (DPA). A, Depression of synaptic responses of a lamina V neurone by microelectrophoretic DPA. The records are pen recordings of the firing rate of the neurone excited alternately by noxious heating (53°C) and by deflection of adjacent hairs. The time of DPA ejection (80 nA) is indicated by the interrupted bar. B, Dose-dependent depression by microelectrophoretic DPA (10, 20, 40 nA) of DPA (5 nA)-induced firing of a lamina V neurone. Depression by glycine (5 nA) is also shown. C, Depression of synaptic excitation of a lamina IV neurone by intravenous DPA. The graph plots the total number of action potentials evoked by each period of noxious heating (53°C) (●) and by deflection of adjacent hairs (○) (ordinate) with respect to time (abscissa).
With 7 neurones DLH-induced firing was depressed by regular ejection of Met\textsuperscript{5}-enkephalin (MENK) for periods of 30-60 sec, and the possible modification by DPA was examined by comparing responses before, during and after DPA ejection. Depression of synaptic responses by MENK was less satisfactory for quantitative purposes since it required ejection of MENK for periods of 4-8 min, making comparisons of MENK effects with or without DPA more difficult. This type of test was performed with 4 neurones responding to noxious heat stimulation.

By either test no evidence was obtained that DPA enhanced the action of MENK. Fig.30A shows results from one experiment in which depression of DLH-induced firing was studied. Glycine ejection was alternated with that of MENK. This use of another depressant is particularly important in experiments of this type to ensure that apparent potentiation of the action of MENK is not associated with a changed effectiveness of glycine. With this neurone the current of DPA was sufficient to depress cell firing but was without effect on depression by MENK or glycine. With the results shown in Fig.30B, MENK (40 nA) depressed the excitation of a neurone by noxious heating of the skin, but added ejection of DPA (60 nA) did not enhance this depression.

**Intravenous DPA:** The possible interaction with the depression of firing by microelectrophoretic MENK was examined with i.v. DPA on 3 neurones. No potentiation was observed (Fig.31A). Since i.v. DPA alone depressed both nociceptive and non-nociceptive responses, the depressant
Fig. 30. Lack of interaction between microelectrophoretic administration of D-phenylalanine (DPA) and met\textsuperscript{5}-enkephalin (MENK).

A, Depression by MENK of DLH-induced firing of a lamina V neurone. DLH (30 nA) was ejected continuously to maintain cell firing. Microelectrophoretic administration of glycine (10 nA) was alternated with that of MENK (30 nA).

B, Depression by MENK of evoked firing. The neurone of lamina IV was excited by alternately by noxious heating of the glabrous skin of a hindlimb digital pad and by deflection of adjacent hairs with a moving air jet. Upper records show depression of these response by MENK (40 nA). Lower records show that ejection of DPA (60 nA) preceded and was continuous with that of MENK.
A

DPA 30nA

-- GLY 10nA --- MENK 30nA

MIN

B

MENK 40nA

DPA 60nA

MENK 40nA

MIN

NOXIOUS HEAT

HAIR DEFLECTION

SPIKES PER SECOND

SPIKES PER SECOND
Fig. 31. Lack of effect of intravenous DPA on depression of cell firing by met⁵-enkephalin (MENK). A, Alternate excitation of a neurone in lamina V by noxious heat (52°C) and a moving air jet. Note that microelectrophoretic administration of MENK (80 nA) depressed both responses. The lower records were obtained 25 min after the intravenous injection DPA (60 mg/kg). B, The histograms show the depression by MENK (80 nA) of nociceptive and non-nociceptive responses of the same neurone calculated as a percentage of control (n=4) both before, and after, DPA administration respectively. The bars indicated standard errors of the means.
action of MENK was calculated as a percentage of controls both before and after DPA administration. By this method no potentiation of MENK was observed (Fig. 31B).

(b) Kelatorphan

As with DPA, kelatorphan was first examined for its effects alone, and then for possible potentiation of inhibition by MENK.

Results were obtained from 21 dorsal horn neurones. On the basis of depth from the dorsal cord surface, 2 neurones were in lamina I, 9 in lamina IV and 10 in lamina V. Fifteen were WDR neurones. Three neurones appeared to nociceptive specific since they responded to noxious but not innocuous cutaneous stimuli. Peripheral stimuli were not tested on the remaining 3 neurones.

(i) Interaction between endogenous opioid peptides and kelatorphan

In view of the finding that inhibition involving opioid peptides is present in the spinal cord of anaesthetized cats subjected to surgery (Duggan et al 1984), it was important to determine if administration in the dorsal horn of kelatorphan, a potent peptidase inhibitor in vitro, altered the evoked responses of dorsal horn neurones.

Kelatorphan-induced depression: With 19 neurones, the effects of kelatorphan administration in the dorsal horn were studied. The effects of 2 or more administrations of kelatorphan were examined with most neurones. With 6 neurones, this compound was ejected (15-70 nA, mean 55 nA,
for 3-41 min) near the cell body and with all 6, gated C responses or firing produced by ejection of DLH (15 nA) were not altered. With other 13 neurones, kelatorphan was administered in the SG. For 8 of 13 neurones, kelatorphan ejection (60-120 nA, mean 100 nA, for 5-10 min) in the SG reduced noxious heat or gated C responses, to a mean of 84% ± 4 S.E.M. (n=8) of control responses (Fig.32,33). Responses to hair deflection were similarly reduced with 2 neurones but unaffected with a third, while spontaneous firing was consistently unchanged. The evoked responses of the remaining 5 neurones were not inhibited by kelatorphan.

**Blockade of kelatorphan-induced depression by naloxone:**
To investigate whether these modest effects of kelatorphan in the SG were due to activation of opioid receptors, naloxone was administered at the same SG site with 7 of the 8 neurones studied. With all 7, naloxone ejection (15-150 nA, mean 105, for 6-10 min) immediately prior to and during kelatorphan administration blocked the inhibitory effect of the latter (Fig.32,33).

**Correlation between the effects of kelatorphan and naloxone in the SG:** If the nociceptive excitation of a particular lamina IV/V neurone is tonically inhibited by an action of enkephalin in the SG, administration of naloxone at this site might be expected to enhance such excitation. Thus for 11 neurones, the effect of kelatorphan administration in the SG was compared with that of independent naloxone administration at the same site. The results are summarized in Table V.
Table V. THE EFFECTS OF ADMINISTRATION OF KELATORPHAN AND NALOXONE IN THE SUBSTANTIA GELATINOSA, 11 NEURONES.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>REDUCTION OF NOCICEPTIVE RESPONSE</th>
<th>ENHANCEMENT OF NOCICEPTIVE RESPONSE</th>
<th>NO EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>KELATORPHAN</td>
<td>7 cells</td>
<td>0 cells</td>
<td>4 cells</td>
</tr>
<tr>
<td>NALOXONE</td>
<td>0 cells</td>
<td>6 cells</td>
<td>5 cells</td>
</tr>
</tbody>
</table>
Fig. 32. Effect of met⁵-enkephalin (MENK), kelatorphan, and naloxone, administered microelectrophoretically in the SG, on the noxious heat and deflection hair responses of a lamina IV dorsal horn neurone. Ejection of MENK in the SG reduced noxious heat responses to about 60% of control responses, while kelatorphan reduced these responses to about 90% of controls. In the presence of continued kelatorphan ejection, the same administration of MENK abolished noxious heat responses. Administration of naloxone at this SG site increased noxious heat responses, and prevented both the inhibitory effect of kelatorphan alone and the potentiated effect of MENK in the presence of kelatorphan.
Fig. 33. Potentiation of the inhibitory effect of met-enkephalin (MENK) by kelatorphan, administered microelectrophoretically in the SG, on the gated C responses of a lamina V dorsal horn neurone. Each plotted point is the mean (n=4) number of action potentials evoked by electrical stimulation of C primary afferent fibres of the ipsilateral tibial nerve (0.3 Hz). Ejection of MENK or kelatorphan in the SG produced small reductions of gated C responses. With co-administration of kelatorphan, the same administration of MENK in the SG almost abolished C responses. Prior and concomitant administration of naloxone at the same SG site blocked this potentiation.
For 6 of the 11 neurones, kelatorphan administration in the SG reduced nociceptive responses (mean 84% ± 6 S.E.M., n=6, of controls) while naloxone administration at the same site enhanced nociceptive responses (mean 120% ± 6 S.E.M., n=6, of controls). One neurone was inhibited by SG administration of kelatorphan but not affected by naloxone, and the responses of the remaining 4 neurones were unaffected by either compound.

(ii) Potentiation of inhibition by exogenous MENK by kelatorphan

These effects of kelatorphan suggested that the nociceptive excitation of some dorsal horn neurones may be tonically but weakly inhibited by an action of opioid peptides in the SG. It was thus important to determine to what extent kelatorphan could potentiate the effect of MENK on dorsal horn neurones.

Administration of kelatorphan in the SG: Firstly, the effects of MENK administered in the SG were studied with 12 dorsal horn neurones. Ejection of MENK (10-150 nA, mean 85 nA, for 1.5-6.5 min) selectively inhibited the nociceptive responses of deeper dorsal horn neurones, confirming previous observations (Duggan et al 1977, 1981). Nociceptive responses were reduced to a mean of 80% ± 4 S.E.M. (n=12) of control responses, with recovery times of 0.5-8 (mean 2.7) minutes (Fig.32,33). Kelatorphan was then administered (60-120 nA, mean 100 nA, for 5-10 min) at the same SG site, and the previous MENK administrations repeated during continued kelatorphan ejection. In the presence of kelatorphan the
inhibitory effect of MENK was markedly potentiated, with nociceptive responses reduced to a mean of 33% ± 8 S.E.M. (n=12) of controls. The recovery times were also prolonged compared with MENK alone: 3-30 (mean 8.7) minutes. Examples are illustrated in Fig.32,33.

With 10 of the 12 neurones, naloxone was also ejected (15-150 nA, mean 90 nA, for 2-15 min) at the same SG site. With 9 neurones, naloxone was ejected prior to and continuous with the added ejection of kelatorphan and MENK. In the presence of the dose of naloxone used, the potent inhibitory effect of combined kelatorphan and MENK administration was blocked. As shown in Fig.32,33, nociceptive responses were 95% ± 4 S.E.M. (n=9) of controls. For the remaining neurones, naloxone ejection, commenced during the potentiated inhibitory effect of MENK, fully reversed such inhibition.

Administration of kelatorphan near cell bodies: Five dorsal horn neurones were studied. Ejection of MENK (50-70 nA, mean 55 nA, for 1-2 min) reduced the nociceptive excitation of all neurones, to a mean of 77% ± 4 SEM (n=5) of control responses, with recovery times of 0.5-7 (mean 2) min.

When kelatorphan was administered (30-70 nA, mean 60 nA, for 4-6 min) prior to and concomitantly with MENK, however, the inhibitory effect of the opioid was potentiated on only 1 of the 5 neurones. In this instance, the gated C response was reduced to 34% of control, an effect which was abolished by prior ejection of naloxone at the same site. For the remaining 4 neurones, nociceptive responses were reduced to a mean of 79% ± 8 S.E.M. (n=4) of controls, a level similar to that obtained with MENK alone.
4.3. DISCUSSION

During the last decade, evidence has accumulated that opioid peptides play an important role in modulating the transmission of nociceptive information in the superficial dorsal horn of the spinal cord. The studies outlined in this chapter provide further new evidence. The opioid peptides and multiple opioid receptors are not only involved in the processing of sensory information in the dorsal horn, but also in the control of motoneurones in the ventral horn.

4.3.1. Opioid Peptides and Control of Nociception

A. Inhibition by metorphamide, an endogenous \( \mu \)-preferring agonist, on dorsal horn neurones

Although there is little doubt that exogenous \( \mu \)-receptor agonists can induce analgesia by influencing the activity of spinal neurones, there is little evidence for endogenous \( \mu \) ligand-induced inhibition in the dorsal horn. The present work has shown that metorphamide, a \( \mu \)-preferring opioid peptide, produces a potent naloxone-reversible inhibition of the nociceptive responses of dorsal horn neurones of the spinal cord in the cat when administered electrophoretically. The presence of an \( \alpha \)-amide residue in this peptide probably makes it less susceptible to breakdown by peptidases (Mains et al 1983), and this may be responsible for the potent effects observed.

It is likely that the effects of metorphamide were due to
an action on opioid $\mu$-receptors. Evidence favouring this
conclusion comes from the similarity between the effects of
metorphamide and microelectrophoretic morphine (Duggan, Hall
& Headley 1977a), effects were readily reversed by naloxone,
and the high affinity of metorphamide for $\mu$-receptors which
are particularly concentrated in the SG (Waksman et al 1986).
This compound was similar to morphine in selectively
inhibiting nociceptive responses of dorsal horn neurones,
suggesting a presynaptic action at primary afferent terminals
(Duggan, kHall & Headley 1977, Davies & Dray 1978, Woolf &
Fitzgerald 1981, Sastry & Goh 1983). Binding studies favour
this conclusion. Using [$^3$H] morphine, $\mu$-opioid binding sites
were observed in the superficial dorsal horn of the rat, and
the marked decrease in the number of binding sites after
transection of the sciatic nerve suggests that these
receptors exist mainly on primary afferent fibres (Fields et
al 1980).

B. Metorphamide and $\kappa$-receptors in the dorsal horn

It is of interest that there are important
differences between the effects of morphine and metorphamide
on cell bodies. The present and previous experiments have
found that morphine rarely inhibits, in a naloxone-sensitive
fashion, the firing of neurones in laminae IV and V of the
dorsal horn in the cat when administered near the bodies of
these neurones. A similar result was reported by Fleetwood-
Walker et al (1985) with another $\mu$-agonist [D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin (DAGO). In the monkey spinal cord, the
effects of morphine on spinothalamic neurones of dorsal horn are variable (Willcockson et al 1986). In contrast, the effects of metorphamide ejected near cell bodies show a non-selective inhibition, although there was often a relative greater reduction of responses to noxious compared with non-noxious skin stimuli. Since metorphamidse also binds to $\kappa$ receptors (Weber et al 1983), it is possible that the affinity of metorphamide for $\kappa$-receptors may underlie these actions. Thus, although this peptide has been proposed as a putative endogenous $\mu$-ligand, the present results suggest that it can interact with $\kappa$-receptors and so be of physiological significance. The findings that a relative high concentration of dynorphin occur in lamina V of the dorsal horn (Cruz & Basbaum 1985), and that electrophoretic administration of dynorphin$^{1-13}$, the $\kappa$ agonist, near cell bodies produced similar effects (Iggo, Steedman & Fleedwood-Walker 1985, Fleedwood-Walker et al 1985) to those produced by metorphamide, seem to bear out this assumption. Compared with administration in the SG where $\mu$ receptors are highly concentrated, metorphamide produced less inhibition of nociceptive responses than when administered near cell bodies in deeper laminae where a high concentration of $\kappa$-receptors are found.

C. Interaction between enzymic inhibitors and Met-enkephalin

Since no uptake systems for enkephalins have been described, it is commonly considered that peptidase-induced
catabolism inactivates synaptically-release enkephalins. Four peptidases have been involved in this process (Fig.10). This rapid degradation has probably frustrated the therapeutic use of neuropeptides. For example, intrathecal administration of enkephalin (400μg) produced only 2-3% of the analgesic effect of morphine (15μg), using tail flick and hot plate tests in the rat (Yaksh et al 1977), and the i.c.v. administration of 33 nmol of morphine produces an effect similar to 670 nmol of Met-enkephalin (670 nmol) (Graf et al 1976).

Numerous pharmacological in vitro and in vivo studies with peptidase inhibitors have shown that these compound can protect enkephalins from breakdown (Schwartz, Costentin & Lecomte 1985).

(a) Kelatorphan

Among these inhibitors, by in vitro tests kelatorphan is the most potent (Fournie-Zaluski et al 1984, Waksman et al 1985). The present studies have shown that when administered in the SG, kelatorphan greatly potentiates the inhibitory effect of exogenously administered Met$^5$-enkephalin (MENK) on the evoked responses of deeper spinal neurones. These findings are in general accord with results of biochemical (Waksman et al 1985) and behavioural experiments (Fournie-Zaluski et al 1984) with kelatorphan. Furthermore, high concentrations of both enkephalin (Hunt et al 1981) and enkephalinase (Waksman et al 1985) occur in the SG. Thus, the present results provide an in vivo neurophysiological
confirmation that in a region of high enkephalinase concentration, enzymic inactivation is a significant factor determining the potency of exogenously-administered enkephalins. Since kelatorphan is a full inhibitor of enkephalin degrading enzymes (Waksman 1985a), inhibiting enkephalinase and aminopeptidase as well as dipeptidylaminopeptidase (Fournie-Zaluski et al 1984, 1985, Waksman et al 1985b, Dickenson 1986), the present evidence that kelatorphan-induced potentiation was fully antagonized by naloxone suggest that degrading enzymes are very important in termination of the physiological effects of enkephalins.

This study further suggests that in the SG of the preparation used there is a tonic release of endogenous opioid peptide(s), the action of which is regulated by enzymic inactivation. The inverse correlation between the effects of kelatorphan and naloxone administered in the SG suggests the presence of tonically active opioid-mediated inhibition of the nociceptive excitation of deeper dorsal horn neurones. High concentrations of both enkephalin (Hunt et al 1981) and enkephalinase (Waksman et al 1986) have been found in the SG, and enkephalin-degrading enzyme systems may thus play an important role in regulating the actions of endogenous opioid peptides in this spinal region. With about 40% of neurones studied, however, both kelatorphan and naloxone were without effect, suggesting that such tonic opioid inhibition is not exerted on all dorsal horn neurones in this preparation. Given the complexity of the synaptic organization in this area such a result is not unexpected.
It is interesting that kelatorphan inhibited dorsal horn neurones and potentiated the depressant effect of exogenous MENK, only when administered in the SG but not near cell bodies. This marked difference does not favour the presence of tonic inhibition involving opioid peptides in the vicinity of dorsal horn neuronal cell bodies, and probably results from a relative paucity of physiologically active opioid peptide-releasing synapses at this region when compared with the SG. Such findings emphasize the advantages of the SG when studying spinal opioid mechanisms, and suggest that in general the effects of opioid administration on single cells in the central nervous system may be more readily related to function when areas containing high enkephalinase concentrations are studied.

(b) D-phenylalanine (DPA)

Apart from the three enzymes cited, carboxypeptidase A has been shown to inactivate Met-enkephalin in vitro (Hughes 1975). If such a process is important in vivo, it would be expected that inhibition of this enzyme would potentiate the action of exogenous enkephalin. This did not occur in the present studies using amounts of D-phenylalanine (DPA), which alone were sufficient to depress cell firing. There are several possible explanations for this apparent lack of interaction between MENK and DPA on the responses of dorsal horn neurones. Firstly, DPA is a weak inhibitor of carboxypeptidase A (Fig.11) ( Ehrenpreis et al 1979), and the local concentrations of DPA achieved in present experiments
may have been too low to inhibit the activity of this enzyme. Behavioural experiments, Giusti et al (1985) also reported that, based on the slight inhibition effect of DPA on carboxypeptidase A, the inhibition of this enzyme and analgesia by DPA might not be directly correlated. Secondly, carboxypeptidase A seems to be relatively unimportant in the catabolism of enkephalins, when compared with aminopeptidase and enkephalinase (Hughes 1983). Particularly, enkephalinase appears to be more specific for the physiological inactivation of enkephalins (Roques et al 1980). Thirdly, if the naloxone-reversible analgesia by DPA in mice and humans (Ehrenpreis et al 1979, 1980) is the consequence of reduced degradation of ENK, then it is possible that this process does not occur at a spinal level. The rather potent analgesia by DPA in a strain of mouse having high pituitary endorphin levels (Cheng & Pomeranz 1979) seems to indicate an interaction between DPA and opioid peptides at supraspinal sites. Another possibility which cannot be excluded, however, is that DPA may be an agonist at sites also acted upon by naloxone. The experiments of Ehrenreis et al (1979, 1980) on DPA-induced analgesia did not report the effects of naloxone alone. If naloxone produced hyperalgesia in these tests then a reversal of the action of DPA does not necessarily indicate that DPA had an action either directly or indirectly at opioid receptors.

The present experiments do not favour an action of DPA at opioid receptors. In the SG, where opioid peptides, such as MENK or metorphamide, depress the responses of deeper dorsal
horn neurones to noxious cutaneous stimuli, DPA was without effect. This point of view was supported by studies of Stone (1983) on neurones of the globus pallidus and cerebral cortex of the rat. Inhibition by DPA, while correlating well with the effect of MENK, was poorly antagonized by naloxone. The depression by DPA of neuronal firing observed in the present experiments may underlie the reduced responsiveness of mice with hot plate testing.

Although studies on the analgesic effects of enzymic inhibitors are still at an early stage, the results to date have indicated that appropriate compounds which inhibit opioid peptide catabolism may offer considerable therapeutic potential. These compounds also promise to be useful pharmacological tools with which to study neuropeptide release in vivo.

4.3.2. Segmental inhibition

A, A possible role of Aδ-afferents in homoafferent nerve-induced inhibition (HMI)

The non-selective nature of HMI in this investigation has similarities to the effects of transcutaneous electrical stimulation (TNS) in humans (Wall & Sweet 1967, Campbell & Taub 1973, Shao, Wu & Zhao 1978, Eriksson 1985). It needs to be pointed out that, as originally proposed, such inhibition was believed to be selective for nociception, but subsequent experiments have not supported this. Shao et al (1978) found that electrical stimulation of a peripheral nerve via
subcutaneous needles or transcutaneous surface electrodes elicited paresthesiae in the innervated region using stimuli adequate to reduce where touch and pain sensibility. At such a current intensity, a single stimulus always evoked a small Aδ wave in the average compound action potential. In the previous cat experiments, excitation of only Aδ fibres in the sural nerve, which was sufficient to fire the dorsal horn neurone studied, produced no HMI whereas the activity of the whole spectrum of A fibres induced potent inhibition (Wu, Chao & Wei 1974). The present work also suggests that the optimal HMI resulted from excitation of all myelinated afferent fibres (Aβ and Aδ). Although the importance of Aβ activity in this inhibition was emphasized by Woolf & Wall (1982), they mentioned that small myelinated afferent fibres (Aδ) were still involved in their study. These results favour the assumption that impulses from fine myelinated afferent fibres (Aδ) play an important role in this inhibition. Intracellular recording from dorsal horn neurones showed that repetitive homoafferent nerve stimulation at intensities sufficient to excite Aδ afferent fibres produced a prolonged depolarization but no action potentials (Wu, Zhao, Chen & Shao 1978), suggesting that this depolarization results in, at least partially, reduced transmission of sensory information in the dorsal horn. This may be similar to the "depolarization block" produced by continued administration of large amount of excitant amino acids in microelectrophoretic experiments. It is necessary to point out that with tetanic stimulation of C afferent
fibres, a powerful prolonged inhibition of nociceptive responses of dorsal horn cells was produced (Chung et al 1984). But the mechanism of this inhibition may differ from that of Aδ afferent-induced inhibition. Thus, care must be used in interpreting clinical and experimental reports of analgesia produced by a variety of peripheral techniques, such as nerve stimulation and acupuncture, since a number of different mechanisms may be involved.

B. Lack of peripheral mechanisms in HMI

In this investigation, homoafferent nerve stimulation (HMS) potently inhibited the nociceptive heat responses of dorsal horn neurones, but did not reduce the discharges produced by heat stimulation in the plantar afferent fibres. This dissociation suggests that a central mechanism is responsible for this inhibition. Campbell & Taub (1973), however, reported that analgesia from electrical stimulation of peripheral nerve resulted from peripheral blockade in humans. They found that when electrical stimulation (50V) was delivered proximally to the digital nerves of the finger, both the touch and pain threshold of the finger were elevated, and that an Aδ wave was always present in the averaged compound action potential recorded from the median nerve during intermittent stimulation but was absent with continuous stimulation. But under such experimental conditions, it is impossible to examine the blockade of C fibres. Further, Zhao et al (1978) reported that HMS at the intensity of Aδ excitation reduced and delayed only Aδ- and
Aδ-waves in the averaged compound action potential of the cat sural nerve, but did not reduce the C wave. These results suggest that the peripheral mechanism has no important role in both the HMI and the TNS-induced analgesia (Swett & Law 1983). If the intensity of HNS is sufficient to excite unmyelinated afferent fibres, then a peripheral mechanism is inevitably involved in contribution to this inhibition. But since such stimulation will evoke severe pain it can scarcely be called an analgesic procedure.

C, HMI and opioid peptides

The observation that microelectrophoretic administration of naloxone in the SG produced a just detectable reduction of HMI is consistent with that of previous reports. Systemic administration of naloxone partially blocked A afferent-induced antinociception, suggesting the involvement of opioid peptides in this inhibitory process (Woolf et al 1977, Sjolund & Eriksson 1979, Woolf, Mitchell & Barrett 1980, Cervero, Schouenbory & Sjolund 1981, Woolf & Fitzgerald 1982). Compared with these results, however, in the present work naloxone had minimal effects on HMI. There are differences between the present and other experiments. Firstly, in other experiments, animals had either intact spinal cords (Woolf et al 1977, 1980) or were decerebrated, with transection of the spinal cord, and without anaesthesia (Cervero et al 1981, Woolf et al 1982). In contrast, the present experiments were performed on spinal anaesthetized cats. It is possible that
in intact preparations, in addition to segmental inhibition, there is also a supraspinally derived inhibition which involved opioid peptidergic interneurones. Consistent with this proposal, the HMI and naloxone-reversible effect in intact animals were more potent than that in spinal animals (Yaksh et al 1977). In addition, Yaksh & Elde (1981) have shown that impulses in C afferent primary fibres produce a release of met$^5$-enkephalin (MENK) in the superfusates of cats whereas no such release of MENK was produced by excitation of A$\beta$ afferent primary fibres. However, the release of MENK was not detected when A$\delta$ afferent primary fibres were stimulated without C fibres excitation. In the present study, the intensity of HMS was sufficient to excite all myelinated afferent fibres of the nerve stimulated (A$\beta$ and A$\delta$). It is possible that met$^5$-enkephalin may be, in some degree, released by excitation of fine myelinated afferent fibres (A$\delta$) and contribute to HMI. Thirdly, the slight naloxone-reversible effect on HMI may indicate that naloxone-insensitive opioid peptides may mediate this inhibition. In view of the finding that i.t. dynorphin produced a potent analgesia (Han & Xie 1982), and this peptide has good affinity for $\kappa$-receptors for which naloxone has relatively poor affinity (Chavkin et al 1982), dynorphin could be involved in HMI. The finding that naloxone failed to block HMI of 6 neurone in lamina I, where dynorphin has a dense distribution (Cruz & Basbaum 1985), is possibly relevant to this assumption. Finally, a simple explanation is that compounds other than opioid peptides are mainly responsible
for this inhibitory process.

D. **Heteroafferent nerve-induced inhibition (HTI) and GABA**

Naloxone failed to reduce the HTI of dorsal horn neurones tested in the present study. In good agreement with the report by Sinclair et al (1980), inhibition of dorsal horn neurones was produced only when Aδ fibres of the contralateral plantar nerve were activated, and this inhibition was unaltered after i.v. injection of naloxone. These results suggest that opioid peptides do not contribute this inhibition. However, Sjolund et al (1979) found that naloxone partially reduced acupuncture-like analgesia only when the acupuncture-like stimulation was at high intensity and low frequency. It is possible that such analgesia was mediated by the release of enkephalins from C afferent fibres (Yaksh & Elde 1981). Possibly relevant to this result, in one neurone when conditioning stimulation was unintentionally applied at an intensity which would excite unmyelinated afferent fibres, this C afferent-induced HTI was reduced by microelectrophoretic administration of naloxone. Except for this case, only Aβ and Aδ afferent fibres were excited by conditioning stimuli in the present experiments.

Since very high concentrations of GABA are present in the superficial dorsal horn (Miyata & Otsuka 1975, Barber et al 1978, Ptrick, McBride & Felten 1983) and bicuculline specifically blocks inhibition by GABA (Curtis et al 1971), the present finding that microelectrophoretic administration
of bicuculline in the dorsal horn, especially in the SG, reduced HTI suggests that GABA-ergic systems contribute to this inhibition. Other investigators have studied the involvement of GABAergic neurones in the inhibition of neurones in the dorsal horn (Game & Lodge 1975, Duggan & Foong 1985). Immunocytochemical studies suggest that the small-diameter primary afferent fibres are presynaptically controlled by spinal GABAergic neurones in the SG (Basbaum, Glazer & Oertel 1981), and electrophysiological evidence showed that GABAergic neurones presynaptically control Ia afferents from muscle (Curtis, Lodge & Brand 1977, Curtis & Malik 1985) and primary cutaneous afferents (Gmelin & Zimmermann 1983). These results suggest that presynaptic GABAergic inhibition possibly is responsible for HTI. On the basis of the finding that two types of GABA receptors (A and B) are probably present on Aδ and C primary afferent fibres (Desarmenien et al 1984), and that the GABA A receptor is bicuculline-sensitive (Bowery 1982), the HTI seems to be, at least partially, associated with the activity of GABA A receptors. The GABA B receptor is bicuculline-insensitive, and the distribution of GABA B binding is concentrated mainly in laminae I and II of the dorsal horn, which differs from the relatively wide distribution of GABA A binding (Price et al 1984). Whether GABA B receptors are involved in HTI is unknown.

In contrast with the HTI, the HMI was not reduced by bicuculline, and thus GABA may not be involved in HMI. As described in the Methods (Chapter 2), however, in the HMI
experiments the conditioning stimulus ceased before starting the test stimulus. In the experiments of Game & Lodge (1975) and Duggan & Froong (1985), the inhibition reduced by bicuculline lasted 50-100 ms. It is thus possible that the action of GABA had decayed before the test stimulus was delivered. Therefore, the possibility of the involvement of GABA in the HMI was not adequately tested.

4.3.3. Tonic inhibition by opioid peptides on motoneurones

Naloxone and (-)-FMN increased all of the reflexes tested in the present work. Enhancement of monosynaptic reflexes could result from an increased release of transmitter from primary afferent terminals or an enhanced excitability of motoneurones. In either case this could result from a direct excitant action of the opioid antagonists or a reduction in the effectiveness of an inhibition. In the case of latter, the inhibition must be tonically present, since monosynaptic excitation of motoneurones occurs before postsynaptic inhibition resulting from the excitation of any inhibitory interneurones activated by the peripheral nerve stimuli.

A. Stereospecificity

Because of the importance of the effects of naloxone on spinal reflexes in determining the possible function of opioid peptides in the spinal cord, the present experiments have examined the effects of intravenous naloxone and another opioid antagonist FMN on a wider range of spinal reflexes
than has previously been examined in the same animal. FMN is a naloxone-like compound, showing little or no agonist activity in tests of opiate action (Kuhn & Stockhaus 1976), and only the (-)-form is an opiate antagonist. The result that both naloxone and (-)-FMN, but not (+)-FMN, increased a wide variety of reflexes in the spinal cat suggest that opioid peptides are involved at some stage. In addition, consideration of the doses of naloxone used in these experiments indicated that it is unlikely that interference with the action of GABA was responsible for the observed increases in reflexes. Although naloxone does reduce GABA-induced inhibition, the IC$_{50}$ (308 µm) is 50 times that of bicuculline (Dingledine, Iversen & Breuker 1978). Since the intravenous doses of bicuculline reducing GABA-induced inhibition in the spinal cord and cerebellum of the cat is 0.2-0.4 mg/kg (Curtis et al 1971), it is extremely unlikely that enhanced spinal reflexes by 0.05 mg/kg of naloxone can be attributed to GABA antagonism. Supporting this, i.v. administered naloxone, in doses known to enhance spinal monosynaptic excitation in the cat, had no effect on GABA-ergic primary afferent depolarization (PAD) and little or no effect on Ia primary afferent terminal thresholds (Curtis, Malik & Leah 1984).

B. Differential actions and possible functions

Naloxone increased reflexes to stimulation of all primary afferent types but of the ascending volleys, only those to stimulation of unmyelinated primary afferents were
increased, suggesting that opioid peptides have differential effects in the spinal cord.

The naloxone-induced increase of a wide variety of reflexes revealed a non-selective inhibition of both flexor and extensor motoneurones. The functional result of such inhibition may be reduce in movement. It would appear to have little role in analgesia. Reduced motor activity is seen in animals as a delayed response to the inflicting of injury (Wall 1979). In the present experiments the surgery necessary to prepare animals for recording may have been the stimulus to release of opioid peptides. Thus, it is not unreasonable to propose that the non-selective inhibition of spinal reflexes revealed by naloxone may be related to restriction of movement after injury.

In contrast, a selective inhibition of cephalad transmission of impulses in unmyelinated primary afferents was revealed by i.v. naloxone. A similar result was observed by Bernatzky et al (1983) in the decerebrated rat. They demonstrated that intrathecal and intravenous naloxone increases the firing of axons in the ALF to impulses in unmyelinated primary afferent fibres, but had no effect on responses to Aβ or Aδ primary afferent fibres. Since most unmyelinated fibres are derived from peripheral nociceptors, this naloxone-induced selective inhibition may be relevant to analgesia.

In support of this notion that the trauma of surgery evoke release of opioid peptides, Yaksh & Elde (1981) demonstrated that the release of immunoreactive ENK-like
substance from the spinal cord of anaesthetized cats was produced only by impulses in C fibres of peripheral nerves.

C. Localization of action

Intracellular recording indicated that EPSPs of motoneurones were increased, by the doses of naloxone increasing spinal reflexes, in the absence of changes in membrane potential and conductance with two thirds of the neurones examined. Thus, it is unlikely that a somatic hyperpolarization mediates the inhibition produced by naloxone. In this study, using parallel pipette assemblies for recording intracellularly from motoneurones while administering naloxone extracellularly, naloxone did not increase depolarizing potentials. Such a result does not favour a tonic release of opioid peptides near motoneurones. Although negative findings cannot be conclusive, all motoneurones studies were depolarized by an excitant amino acid and the currents used to eject naloxone were relatively large. It is not possible to exclude a remote dendrite or presynaptic inhibition entirely since in the cat the dendrites of motoneurones can extend for up to 2 mm (Brown 1981).

When ENKs have been administered electrophoretically near the terminals of primary afferent fibres on motoneurones, the results have also not favoured a release of opioid peptides near motoneurones as being responsible for the increase in reflexes produced by opioid antagonists. Electrophoretic met-enkephalinamide (MENKA) had no effect on the monosynaptic
excitation of Ia interneurones in the intermediate nucleus, and, although having inconsistent effects on terminal excitability, this opioid peptide reversibly reduced PAD of extensor muscle Ia primary afferent fibres induced by impulses in low threshold afferents of flexor muscles (Curtis, Malik & Leah 1984). A reduction of PAD by MENKA has also been observed with tooth pulp afferents and stimulation in the medullary raphe (Lovik 1983). Such a mechanism, if tonically active, however, would not decrease reflexes in extensor muscles.

The combination of lack of effect by opioid peptides and by naloxone does suggest that the tonic inhibition of motoneurones present in anaesthetized cats does not occur by release of opioid peptides near motoneurones. Rather it is more probable that these compounds control the activity of interneurones, some of which finally inhibit motoneurones or reduce the release of excitatory transmitters from afferent fibres synapsing on motoneurones.

Since naloxone combines with both µ and δ opioid receptors and, with a lower affinity to κ receptors (Kosterlitz 1985), and (-)-FMN has a considerable affinity for both µ and κ opioid receptors, it is difficult to define which receptor and peptide are mainly involved. The evidence that a complex release of opioid peptides, including met-ENK lysine6 and dynorphin derivatives, follows peripheral stimulation of C afferent fibres (Nyberg, Yaksh & Terenius 1983) suggests the possible involvement of several opioid peptides in the tonic inhibition on motoneurones.
5.1. INTRODUCTION

Inhibitory control of spinal transmission from the brain stem has been studied extensively (for review see Lundberg 1982). In the field of nociception, since electrical stimulation (Reynolds 1969, Mayer et al 1971) and microinjection of opiates (Tsou & Jang 1964) in the midbrain was shown to produce profound analgesia, there has been a surge of interest in descending modulation of the activity of dorsal horn neurones responding to peripheral noxious stimuli. A number of brain areas, such as the periventricular-medial hypothalamus (Carstens 1982), the medial preoptic septal areas (Carstens, Mackinnon & Guinan 1982), the periaqueductal grey matter (PAG), the locus coeruleus (LC), the nucleus raphe magnus (NRM) and the lateral reticular nucleus of medulla (LRN) have been demonstrated to influence the responses of dorsal horn neurones of the spinal cord to peripheral noxious stimuli. Numerous reviews support such systems (Basbaum & Fields 1978, 1984, Yaksh & Rudy 1978, Duggan 1982, Bjorklund & Skagerberg 1982, Mayer 1984). The present study will be restricted to an examination of the inhibition of dorsal horn neurones produced by stimulation of the periaqueductal grey matter (PAG) and the dorsolateral pons (DLP) including the locus coeruleus (LC) and the nucleus Kolliker-Fuse (NKF).
5.1.1. PAG-induced inhibition of dorsal horn neurones and the pathways involved

Analgesia has been produced by electrical stimulation in the midbrain periaqueductal grey (PAG) region of a number of species, including rats (Reynolds 1969, Mayer et al 1971, Yaksh, Young & Rudy 1976), rabbits (Zhao, Shao & Yang 1978), cats (Liebeskind et al 1973, Melzack & Melinkoff 1974, Oliveras et al 1974), monkeys (Hayes et al 1979) and humans (Adams 1976, Hosobuchi, Adams & Linchitz 1977). Inhibition in the spinal cord may be an important component of this analgesia since stimulation in the PAG reduced the excitation of lumbar dorsal horn neurones by noxious cutaneous stimuli in anasthetized rats (Bennett & Mayer 1979, Carstens & Watkins 1986), cats (Oliveras et al 1974, Carstens, Yokota & Zimmermann 1979, Zhao, Yang & Yang 1982, Duggan & Morton 1983) and monkeys (Hayes et al 1979). The effects of PAG stimulation may be widespread, however, including inhibition of brainstem neurones excited by peripheral noxious stimuli (Yaksh & Rudy 1978). When opiates have been microinjected into the PAG, antinociception (Yaksh & Rudy 1978, Gebhart 1982) and inhibition of nociceptive neurones in the dorsal horn of spinal cord (Bennett & Mayer 1979, Clark, Edeson & Ryall 1983, Gebhart et al 1984) have been observed. Consequently, opiate analgesia and stimulation-produced analgesia have been proposed to activate common neural pathways to the spinal cord (Mayer & Price 1976).

Electrical stimulation or opiate microinjection in the

Raphespinal neurones in the nucleus raphe magnus (NRM) were excited by electrical stimulation or opiate microinjection in the PAG (Fields & Anderson 1978, Lovick, West & Wolstencroft 1978, Mason, Strassman & Maciewicz 1985). Taken together with the anatomical findings, that direct projections from the PAG to lumbosacral levels are few (Kuypers & Maisky 1975, Kneisley, Biber & Lavail 1978, Castiglioni, Gallawy & Coulter 1978, Basbaum & Fields 1979) (but see Manthy & Peschanski 1982) and there are direct projections from the PAG to the NRM (Gallagher & Pert 1978, Abols & Basbaum 1981), the effects of PAG stimulation have been proposed to be relayed to the spinal cord via neurones of the medullary raphe (Basbaum & Fields 1978).

Although the role of these midline structures of the brainstem in supraspinal inhibition of dorsal horn neurones has been emphasized by such studies, the following findings are incompatible with such an exclusive descending pathway. Firstly, blockade of the NRM by the microinjection of lidocaine failed to affect the PAG-induced inhibitions of noxious heat responses of spinal dorsal horn neurones in the cat (Gebhart et al 1983), and of the nociceptive tail flick reflex in the rat (Sandkuhler & Gebhart 1984). In addition, acute lesions of the raphe region have no effect on morphine-
induced analgesia in the rat (Proudfit 1981). Secondly, naloxone reduced the behavioral analgesia produced by raphe stimulation in the cat (Oliveras et al 1977) and rat (Satoh et al 1980, Zorman et al 1982), and also reduced NRM-induced inhibition of spinal dorsal horn neurones (Chao, Yang & Yang 1981, but see Duggan & Griesmith 1979) and medullary dorsal horn neurones in the cat (Sessle et al 1981). Naloxone, however, had no significant effect on PAG-induced inhibition in these electrophysiological studies. Thirdly, the nociceptive excitation of many dorsal horn neurones is tonically inhibited from supraspinal sites in anaesthetized rats (Necker & Hellon 1978), cats (Besson, Guilbaud & Le Bars 1975, Handwerker, Iggo & Zimmermann 1975, Duggan et al 1977, Soja & Sinclair 1983) and monkeys (Willis, Haber & Martin 1977). In the anaesthetized cat the PAG and medullary raphe do not contribute to this powerful descending inhibition which is reduced or abolished by brainstem lesions only when made bilaterally in the ventrolateral medulla, in the region of the caudal lateral reticular nuclei (LRN) (Hall et al 1981, 1982). Additionally, as with the medullary raphe, electrical stimulation in these lateral regions also inhibits the responses of dorsal horn neurones to impulses in unmyelinated primary afferent fibers (Morton, Johnson & Duggan 1983).

In summary, these results suggest that the NRM may take part in the PAG-induced inhibition of dorsal horn neurones but a NRM-spinal pathway may not be the only descending system influencing these neurones. Thus, one object of the
experiments of this thesis was to investigate the relative contributions of the NRM and the LRN to the inhibition produced in the dorsal horn by PAG stimulation.

5.1.2. Stimulation of the dorsolateral pons (DLP)

A. LC-induced inhibition of dorsal horn neurones

The locus coeruleus (LC) is a small pontine structure, which overlaps the A6 area of noradrenaline containing neurones and is located adjacent to the fourth ventricle. This nucleus has been considered to be involved in such diverse functions as respiration, micturition, stress, sleep, learning (for review see Amaral & Sinnaman 1977, Mason 1984). However, the involvement of the LC in analgesia has been studied only recently. Electrical stimulation of LC regions reduced spinal reflexes to peripheral noxious stimuli in the rat (Segal & Sanberg 1977, Margalit & Segal 1979, Jones & Gebhart 1986). When this area was destroyed there was no change of the baseline nociceptive threshold, and antinociception by i.t. administration of clonidine was enhanced in the rat tail flick test (Ossipov, Chatterjee & Gebhart 1985). The LC has thus been suggested to participate in the descending inhibition of spinal nociceptive transmission, and that this inhibition may be mediated in the spinal cord by $\alpha_2$-adrenoceptors located postsynaptically with respect to NA terminals of descending LC efferent fibers. In support of this, stimulation of the LC inhibited the responses of dorsal horn neurones.

B. Possible involvement of noradrenaline (NA) in inhibition in the dorsal horn from DLP stimulation

(a) NA-containing neurones

The dorsolateral pons (DLP), including the nucleus locus coeruleus (LC), nucleus subcoeruleus, the Kolliker-Fuse nucleus (NKF) and the parabranchial nucleus have a dense concentration of NA-containing neurones (Dahlstrom & Fuxe 1964, 1965, Chu & Bloom 1974, Jones & Moore 1974, Poitras & Parent 1978).

(b) NA-containing terminals from the DLP in the spinal cord

A number of anatomical studies have demonstrated that a direct coeruleospinal pathway exists in the rat (Nygren & Olson 1977, Satoh et al 1977, Westlund et al 1983), cat (Hancock & Pougerosse 1976, Kuypers & Maisky 1977, Stevens, Hodge & Apkarian 1982), monkey (Hancock & Pougerosse 1976, Westlund et al 1984) and human (Papez 1925). Further, there is a relatively dense concentration of NA-containing terminals in the superficial dorsal horn of the spinal cord (Dahlstrom & Fuxe 1965, Kerr 1975). Although the exact origins of all of these fibers is in dispute, in the rat they are derived mainly from the LC (Martin et al 1979, Moore & Bloom 1979, Bjoklund & Skagerbeg 1982, Westlund et al 1983).
After complete bilateral lesions of the LC and the subcoeruleus of the rat, 90% of CA-containing terminals disappeared in the ventral horn, the spinal intermediate zone of grey matter and the ventral part of the dorsal horn. In the superficial dorsal horn, CA-containing terminals were reduced by only 25-50% (Nygren & Olson 1977). In contrast, in the cat, many NA-containing terminals are derived from neurones of the NKF, a structure more lateral and ventral to the LC (Satoh et al 1977, Stevens et al 1982).

(c) Release of NA from the spinal cord

In the cat, the concentration of NA in a spinal superfusate was elevated by electrical stimulation of the sciatic nerve at an intensity adequate to activate C fiber afferents. This increase was abolished by cold block of the cervical spinal cord, or by spinal transection, suggesting that impulses in peripheral nociceptor fibers resulted in the release of NA in the spinal cord from axons of supraspinal neurones (Tyce & Yaksh 1981). In support of this proposal, a release of [³H]NA into the perfused central canal of the cat lumbosacral spinal cord was produced by stimulation of descending tracts, but not by stimulation of dorsal roots (Jones, Umney & Webster 1983). In addition, electrical stimulation of the LC increased the content of metabolites of NA in the rat spinal cord (Crawley, Roth & Mass 1979).

(d) Action of NA in the spinal cord

In the rat, the intrathecal administration of NA
inhibited spinal reflexes to peripheral noxious stimuli, and NA antagonists decreased the nociceptive threshold (Reddy, Maderdrut & Yaksh 1980, Howe, Wang & Yaksh 1983, Sagen & Proudfit 1984). In addition, the role of descending noradrenergic fibers in morphine analgesia has been studied (Kuraishi et al 1979, Yaksh 1979, Hu, Zhong & Tsou 1984). Morphine analgesia was significantly reduced when NA in the spinal cord was depleted with i.t. injection of the neurotoxins, such as 6-DHT (Kuraishi et al 1983) or DSP4 [N(2-chloroethyl)-N-ethyl-2-bromo-benzylamine] (Zhong, Ji & Tsou 1985). When administered electrophoretically near dorsal horn neurones, NA inhibited excitation by cutaneous noxious stimuli (Engberg & Ryall 1966, Belcher, Ryall & Schaffner 1978). When administered in the substantia gelatinosa, NA powerfully suppressed the nociceptive responses of deeper dorsal horn neurones in laminae IV and V (Headley, Duggan & Griersmith 1978). In in vitro slices of the upper dorsal horn of the rat, NA produced a dose-dependent hyperpolarization of SG interneurones in association with a fall in input resistance. The NA-induced hyperpolarization was due to a direct postsynaptic action, since it persisted in low Ca++ and high Mg++ solution or in a medium containing Co++ and high Mg++ (North & Yoshimura 1984).

(e) Involvement of alpha-2 receptor

By using a range of agonists and antagonists, the studies employing intrathecal and electrophoretic
administration of drugs have concluded that the receptors for NA-mediation of the inhibition of transmission of nociceptive information are of the alpha-2 variety (Howe, Wang & Yaksh 1983, Davies & Quinlan 1985, Fleetwood-Walker et al 1985). Under in vitro conditions, North & Yoshimura (1984) showed that NA-induced hyperpolarization of SG neurones was antagonized reversibly by yohimbine and phentolamine, but not by prazosin or by propranolol. Clonidine mimicked the effect of NA, whereas isoproterenol was inactive. These results indicated that NA hyperpolarizes SG neurones through alpha-2 adrenoceptors. A recent finding demonstrated that intrathecal administration of yohimbine reduced the inhibition of the rat tail flick responses produced by stimulation in the LC (Jones & Gebhart 1986). These results are supported by the localized binding of the alpha-2 agonist, paraaminoclonidine, in the upper dorsal horn when using autoradiographic methods (Young & Kuhar 1980, Unnerstall, Kopajtic & Kuhar 1984).

All of these results seem to indicate that NA has a role in the descending control of the dorsolateral pons on spinal transmission of nociceptive information.

However, results contrary to this proposal have been reported. If the effects of stimulation in the LC are produced by release of NA in the spinal cord, then it is important that there be a similarity between the actions of exogenously administered NA and stimulation in the LC. The effects of electrical stimulation of the LC, however, have
been found to be variable, with both selective (Mokha, McMillan & Iggo 1985) and non-selective inhibition (Hodge et al 1981) being observed when studying the excitation of neurones of the dorsal horn by noxious and non-noxious peripheral stimuli. This contrasts with the nearly uniform reports of selective inhibition of nociceptive responses produced by administering NA near spinal neurones (Headley, Duggan & Griersmith 1978, Davies & Quinlan 1985). In addition, an important test of the involvement of NA in LC-induced inhibition of dorsal horn neurones of the spinal cord is whether procedures which alter the release or actions of NA in the spinal cord modify the effects of stimulation in the LC. Hodge et al (1981) reported that significant depletion of NA in the spinal cord by intrathecal administration of 6-hydroxydopamine had no measurable effect on the inhibition of dorsal horn neurones by stimulation in the LC.

With the demonstration that idazoxan, a relatively specific alpha-2 antagonist (Berridge et al 1983, Dettmar, Lynn & Tulloch 1983, Doxey, Roach & Smith 1983, Freedman & Aghajanian 1984), reliably blocked the actions of NA on spinal dorsal horn neurones (Davies & Quinlan 1985, Fleetwood-Walker et al 1985), it seemed appropriate to examine the effect of this drug on inhibition of spinal neurones by stimulation of the dorsolateral pons of the cat.
5.2. RESULTS

5.2.1. PHYSIOLOGY OF DESCENDING INHIBITION

A. The Roles of Medullary Region in PAG-induced Inhibition of Dorsal Horn Neurones

To investigate the relative contributions of medullary regions to inhibition produced in the dorsal horn of spinal cord by stimulation of the periaqueductal gray matter (PAG), PAG-induced inhibition was first measured and then the effect of coagulating medial and lateral areas of the medulla was examined. Thirteen dorsal horn neurones of the spinal cord were studied. They were located in laminae I (one cell), III-IV (9) and V (3), based on the depth from the dorsal surface. In each experiment a PAG site was selected where electrical stimulation produced significant inhibition of the C responses of these neurones.

(a) PAG-induced inhibition

Stimulation in the PAG produced a selective inhibition of the excitation of all 13 WDR dorsal horn neurones of the spinal cord by impulses in primary afferent C fibers. This inhibition was characterized by a rapid onset and offset, which is consistent with previous observations (Duggan & Morton 1983, Zhao, Yang & Yang 1983). An example is shown in Fig.34. Stimulation in the medial PAG with currents at 50 and 100 μA inhibited the gated C responses of a dorsal horn neurone (Fig.34C).
Fig. 34. Stimulation in the periaqueductal grey matter (PAG) produced inhibition of the excitation of a dorsal horn neurone by impulses in primary afferent C fibres.

A, Diagrams showing how the gated C responses were measured. The tibial nerve was electrically stimulated at the start of each oscilloscope sweep. Note that only those action potentials with latencies and stimulus thresholds appropriate to stimulation of unmyelinated fibres are included in the gate (upper), and these gated counts (C responses) are continuously displayed on a pen recorder (lower).

B, PAG stimulation consisted of a tetanus of 0.2 ms pulses at 310 Hz for 100 ms. The last pulse of the train occurred within 10 ms of the start of the counting gate.

C, Stimulation of the PAG (50 µA and 100 µA) inhibited the gated C responses of a dorsal horn neurone.
(b) Lesions of the NRM region

With the array of 4 coagulating electrodes centred initially on the midline at Horsley-Clarke co-ordinates AP-7, V-9, L0, this area, which includes the NRM, was coagulated at 1 mm intervals over its dorso-ventral extent. The regions 1 mm on either side of the midline were then similarly destroyed. This coagulation in 3 parasagittal planes was then repeated rostrally with the electrodes centred on AP-5, and caudally, centred on AP-9.

PAG-induced inhibition was measured with a range of stimulus currents both prior to and following the introduction of the coagulating needles into the brainstem. This was done to take into account the possibility that trauma produced by needle placement in the medulla prior to coagulation may affect the inhibition of dorsal horn neurones evoked by PAG stimulation.

(i) Reduction of C response: introduction of the coagulating needles into the midline decreased the C responses of dorsal horn neurones in 6 of 7 experiments (mean reduction 47 % ± 6 SEM (n=6)), while no change was observed in one. The level of C responses attained during spinal cold block, however, was unchanged, indicating that this decrease in the basal C response was due to increased descending inhibition, presumably through exciting brainstem neurones by mechanical deformation. This effect is shown in Fig.35A.

After lesions, C responses of dorsal horn neurones were further reduced (Fig.35B).
(ii) Slight reduction of PAG-induced inhibition: in 6 of 7 experiments, such lesions of the medullary midline slightly reduced the inhibition of C responses of dorsal horn neurones produced by PAG stimulation. There was no change in one experiment. For each experiment, the reduction in inhibition was calculated by subtracting the inhibition (expressed as a percentage of control C responses) for a given stimulus current obtained following midline lesions from that observed prior to such lesions. These differences in percent inhibition were averaged, the mean reduction for the 6 experiments being 28.3% ± 5.4 SEM (n=6). In no instance was inhibition from PAG stimulation abolished by midline lesions of the medulla.

Results from one experiment are illustrated in Figs. 35 and 36. Prior to any lesion, stimulation (200 µA) in the ventral PAG reduced the gated C response of the lamina IV neurone from 13.6 ± 0.2 S.E.M. to 6.5 ± 0.2 S.E.M. (n=11), a reduction of 7.1 action potentials (52%). Insertion of the coagulating electrodes into the raphe (midline) decreased the uninhibited response to 7.8 ± 0.2 S.E.M., and PAG stimulation then reduced the response to 3.0 ± 0.2 S.E.M. (n=11), a reduction of 4.8 action potentials (62%) (Fig. 35A). Following extensive midline and para-medial coagulation, PAG stimulation was less effective, reducing the C response from 7.0 ± 0.2 to 4.7 ± 0.2 S.E.M. (n=11), a reduction of 2.3 action potentials, or 33% (Fig. 35b). The extent of midline destruction is illustrated in transverse sections, both through the fixed brain stem (Fig. 35D) and in a paraffin
Fig. 35. The effect of coagulation in the medulla on the inhibition of a lamina IV neurone produced by electrical stimulation in the PAG. The recordings show the number of action potentials evoked by impulses in C fibre primary afferents following electrical stimulation of the ipsilateral tibial nerve at 0.6 Hz. The black bars indicate the periods of stimulation in the ventral PAG (AP 0, V 0) by a tetanic train (thirty-seven 0.2 ms pulses at 310 Hz, 0.6 Hz) at the stimulus currents shown, and the periods of cold block of spinal conduction (L1) during which C responses were enhanced by removal of tonic descending inhibition.

A, Inhibition of C responses by PAG stimulation, and reduction of basal C responses by insertion of coagulating needles into the medullary raphe.

B, Inhibition of C responses by PAG stimulation following extensive coagulation in the midline (raphe) and paramedial areas (Figs. 35D, 36A).

C, Inhibition of C responses by PAG stimulation following midline coagulation and limited lesions in the LRN regions. Following the lateral reticular lesions (Fig. 2B), the basal C responses were increased to the level attained during spinal cord block.

D, The photograph shows the extent of midline destruction exposed by a transverse section through the fixed brain-stem at approximately Horsely-Clarke AP -10
A  
CONTROLS

B  
AFTER RAPHÉ LESIONS

C  
AFTER L.R.N. LESIONS

D  
AP. 10
section (Fig. 36), at approximately midway through the longitudinal extent of the lesion. As shown in these figures, haemorrhage was often associated with such lesions.

A possible reason for the limited effectiveness of this procedure in reducing inhibition from PAG stimulation could be inadequate destruction of midline structures including the raphe. Histological examination of the brainstems revealed, however, that in only one instance was the midline lesion considered incomplete, this being the experiment where inhibition from PAG stimulation was unchanged.

The uninhibited C response was not elevated by midline destruction in these experiments, confirming the previous finding that this region does not contribute to tonic descending inhibition in the anaesthetized cat.

(c) Lesions of the LRN region

(i) Increase of C response: bilateral destruction of the LRN regions increased the basal C responses, confirming previous results (Hall, Duggan & Morton 1982). This increased the number of action potentials evoked by each tibial stimulus. In the experiment illustrated in Fig. 35, ipsilateral coagulation of the LRN region after raphe destruction increased the basal C response from 8.3 ± 0.2 S.E.M. (Fig. 35B, last trace) to 19.0 ± 0.3 S.E.M. (n=11) (Fig. 35C, first trace) action potentials, and a further increase in the basal C response, to 26.4 ± 0.4 S.E.M. (n=11), occurred with positioning of the electrodes in the contralateral LRN region.
(ii) Abolition of PAG-induced inhibition: bilateral lesions of the caudal LRN markedly reduced or abolished inhibition from PAG stimulation in 9 of 10 experiments. In 5 cases the LRN regions alone were destroyed whereas in the other 5 the medullary midline was lesioned prior to the lateral lesions. The results differed between the two groups of experiments. When the LRN regions alone were destroyed, the inhibition from PAG stimulation was abolished in one experiment, significantly reduced in 3 and unchanged in one, whereas coagulation of these lateral reticular regions after midline destruction abolished the inhibition in all 5 experiments.

After lesions of LRN regions, PAG-induced inhibition was superimposed on elevated C responses and this C response increase alone may have altered the apparent effectiveness of PAG stimulation. Some of results, however, argue against this explanation. As shown in Fig.35, despite the elevation of C responses (Fig.35C, first trace), the effectiveness of PAG stimulation (200 µA), 31% inhibition, was similar to pre-lesion controls (33% inhibition, Fig.35B, last trace). The absolute number of action potentials removed from the C response by PAG stimulation was even increased (5.8 ± 0.4 compared with 2.3 ± 0.1 S.E.M. previously). In view of the lack of effect of unilateral lesions on PAG-induced inhibition, despite a considerable elevation of the basal C responses, it is extremely unlikely that the lesser rise in basal responses following the second lesion was responsible for the abolition of PAG-induced inhibition. Fig.36
illustrates the extent of destruction observed histologically in a transverse paraffin section of the caudal brainstem from this experiment, with a small area of destruction in the contralateral LRN caused by electrode placement, and the larger area of coagulation in the ipsilateral LRN region. In another experiment, although basal C responses were elevated considerably by abolition of tonic inhibition, stimulation of PAG still produced inhibition which was not abolished until the second LRN region was coagulated. Since this coagulation did not elevated the C response further, the abolition of inhibition from PAG stimulation cannot be attributed to an altered level of C response.

It needs to be emphasized that lesions in the LRN region abolishing inhibition from PAG stimulation were relatively small compared with the extensive destruction of midline area (Fig.36). If a lesion is defined as that produced by the passage of current between the 3 pairs of electrodes, then it was common to abolish PAG-induced inhibition by one or two lesions of each lateral area. By contrast, as many as 45 such lesions were usually performed in destroying the midline, and para-medial areas, with relatively modest effects on inhibition from the PAG.

Fig.37 shows results from one experiment where lateral reticular lesions alone significantly reduced PAG-induced inhibition. Stimulation in the ventral PAG at 100 µA reduced the gated C response of the lamina IV neurone by $19.7 \pm 1.7$ S.E.M. (n=6) spikes (41% inhibition), and at 150 µA, by $30.6 \pm 1.9$ S.E.M.(n=5) spikes (59% inhibition). After bilateral
Fig. 36. Histological results from the experiment illustrated in Fig. 35. The extent of destroyed tissue apparent in the 15 µm transverse paraffin sections through the brainstem at approximately Horsley-Clarke AP -10 and AP -16 have been marked as stippled areas on the diagrams of these sections. The terminology of Berman (1968) has been used. CI, inferior central raphe nucleus; FTG, gigantocellular tegmental field; FTL, lateral tegmental field; INF OL, inferior olive; V4, fourth ventricle; LRN, lateral reticular nucleus; TRIGEM, spinal trigeminal nucleus; CUN, cuneate nucleus; XII, hypoglossal nucleus.
Fig. 37. The effect of bilateral coagulation in the LRN regions of the medulla on the inhibition of a lamina IV neurone by electrical stimulation in the PAG. As in Fig. 35, the recordings show the number of action potentials evoked by impulses in C fibre primary afferents following electrical stimulation of the ipsilateral tibial nerve at 0.23 Hz. The black bars mark the periods of stimulation in the ventral PAG (AP +2, V +1) by a tetanus train (thirty-one 0.5 ms pulses at 310, 0.23 Hz) at the stimulus currents shown, and the periods of cold block of spinal conduction (L1).

A, Inhibition of C responses by PAG stimulation and enhancement of C response by removal of tonic descending inhibition during spinal cold block.

B, Inhibition of C responses by PAG stimulation and the remaining tonic inhibition following bilateral coagulation in the LRN regions.
A  

CONTROLS

GATED C FIBRE RESPONSE

STIM P.A.G.
100µA

150µA

COLD BLOCK

0 100 SEC

0 100 SEC

0 3 6 MIN

B

AFTER L.R.N. LESIONS

GATED C FIBRE RESPONSE

100µA

150µA

COLD BLOCK

0 100 SEC

0 100 SEC

0 3 6 MIN
coagulation of the LRN regions, stimulation at 100 μA removed only 5.0 ± 2.5 S.E.M. (n=6) spikes (7% inhibition), and 150 μA, 10.7 ± 1.7 S.E.M. (n=7) spikes (15% inhibition). Tonic descending inhibition as revealed by cold block was reduced but not abolished by these lesions.

B. Spinal Inhibition from Stimulation in the Region of the LC and NKF

In these experiments the stimulating electrodes were initially positioned dorsal to the LC and NKF and stimulation performed at progressively more ventral sites using a range of stimulus currents at each site. A total of 40 neurones were studied and 119 sites were stimulated in the dorsolateral pons (Fig.38).

(a) LC-induced inhibition

An important result of these experiments was that, unlike stimulation at other sites in the dorsolateral pons, stimulation in the region of the LC selectively inhibited nociceptive responses of 8 out of 9 neurones of laminae IV and V. Excitation of by hair deflection was unaffected. This selective effect of stimulation in the LC region is shown in Figs.39 and 40. Inhibition was readily produced with currents of 50 to 300 μA (mean of 164 μA ± 13 S.E.M., n=31). As shown in Fig.41, the degree of inhibition clearly depended upon both frequency and intensity of LC stimulation.

(b) NKF-induced inhibition
Fig. 38. Inhibition of dorsal horn neurones by electrical stimulation in the dorsolateral pons. The marked sites were stimulated electrically by a tetanic train (0.3 ms pulses, 300 Hz, 20-100 ms) while recording from an ipsilateral lamina IV or V neurone excited by impulses in C fibre primary afferents following electrical stimulation of the ipsilateral tibial nerve or by noxious heat. Filled symbols: inhibition of dorsal horn neurone; Open symbols: no effect. The terminology of Berman (1968) has been used. BC, brachium conjunctivum; CAE, nucleus caeruleus (locus coeruleus LC); FTG, gigantocellular tegmental field; FTP, paralemniscal tegmental field; KF, Kolliker-Fuse nucleus; LLV, ventral nucleus of the lateral lemniscus; MLB, medial longitudinal bundle; P, pyramidal tract; V4, fourth ventricle. TAD, accessory dorsal tegmental nucleus.
Fig. 39. Inhibition of a dorsal horn neurone by electrical stimulation of the sites of the dorsolateral pons. The recording shows that the total number of action potentials evoked by noxious heat ($50^\circ$) and deflection of hairs was determined with a microprocessor-controlled integrator which sampled and subtracted spontaneous firing. Note that only stimulation of the LC (site A) produced selective inhibition of noxious heat responses.
Fig. 40. Selective and non-selective inhibition of the responses of a dorsal horn neurone from electrical stimulation in the locus coeruleus (LC) and the Kolliker-Fuse nucleus (NKF). The histograms show LC stimulation (200 µA) selectively inhibited the gated C responses of a dorsal horn neurone excited by impulses in primary afferent C fibres following electrical stimulation of the tibial nerve, whereas NKF stimulation (200 µA) inhibited both the gated C responses and hair deflection responses. The right diagram illustrates the distribution of the selective inhibitory and non-selective inhibitory regions. Note that stimulation of the LC region mainly produced selective inhibition.
Fig. 41. Inhibition of the gated C responses by electrical stimulation of the LC and the NKF. The pen records show that the C responses of a lamina V neurone were evoked by impulses in primary afferent C fibres following electrical stimulation of the tibial nerve. Note frequency-dependant inhibition by NKF stimulation (10-500 Hz at 100 µA) (the upper record), and intensity-dependent inhibition by stimulation of the LC and the NKF (100-300 µA at 300 Hz) (the lower record).

### TABLE VI COMPARISON BETWEEN LC- AND NKF-INDUCED INHIBITION

<table>
<thead>
<tr>
<th>SITE</th>
<th>CELL NUMBERS IN INHIBITION OF C-RESPONSE OF</th>
<th>(percentage of control)</th>
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<tr>
<td></td>
<td></td>
<td>MORE THAN 70%</td>
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<tr>
<td>LC</td>
<td>2</td>
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<tr>
<td>NKF</td>
<td>9</td>
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In contrast to the effects of stimulation in the LC, non-selective inhibition of dorsal horn neurones resulted from stimulation in the region of the NKF (Fig. 39, 40) with currents of 50-300 µA (mean 157 µA ± 18 S.E.M., n=19).

With 11 neurones the effects of identical currents for stimulation in the LC and the NKF were compared. In all cases greater inhibition of C-responses was produced by NKF stimulation (mean inhibition of 76.3% ± 7.5 S.E.M.) than by stimulation in the LC (mean inhibition of 56.8% ± 6.7 S.E.M.). Table VI compares the extent of inhibition of C-responses by the same intensity of LC and NKF stimulation (50-200 µA).

Because electrical stimulation can stimulate fibres of passage as well as neurones at the site stimulated, and it was an unexpected result that stimulation at two sites containing catecholamine-containing cells gave qualitatively different inhibition in the spinal cord, 6 neurones were studied when L-glutamate was microinjected (0.1-0.2µl) in the region of the LC and NKF. Despite the effectiveness of electrical stimulation, L-glutamate, microinjected in the NKF, had no effect on the evoked responses of the 3 spinal neurones studied. By contrast, with 2 of 3 neurones (all inhibited by electrical stimulation), L-glutamate microinjection in the LC also reduced C-responses.

5.2.2. PHARMACOLOGY OF DESCENDING INHIBITION

An important test of the involvement of noradrenaline (NA) in the spinal effects of electrical stimulation in the
LC and the NKF is whether procedures which alter the release or actions of NA in the spinal cord modify the effects of stimulation. First it was necessary to examine whether idazoxan, a specific α2-antagonist, blocked the actions of NA and clonidine on spinal neurones, and then to test whether idazoxan modified LC- and NKF-induced inhibitions.

A. Blockade of NA-induced inhibition of spinal nociceptive transmission by idazoxan

Recordings were obtained from 25 wide dynamic range (WDR) neurones excited by both noxious and non-noxious peripheral stimulation. Based on depth from the dorsal surface, neurones studies were distributed in laminae I (one cell), III (one), IV (10) and V (13).

(a) Inhibition by Noradrenaline and Clonidine

In confirmation of previous reports (Headley, Duggan & Griersmith 1978), microelectrophoretic ejection of NA (15-70nA, mean 30 nA, for 0.5-4 min) in the substantia gelatinosa (SG) reduced the excitation of neurones of laminae IV and V by noxious heating of the skin or by impulses in unmyelinated primary afferents (11 neurones). Noradrenaline (15-20 nA, mean 18 nA for 1-7 min) also reduced these responses when ejected near cell bodies (5 neurones) (see Table VII). There was no apparent difference in the extent of inhibition produced by the administration of NA with similar currents in the SG and near cell bodies. Fig.42A shows an example of NA-induced inhibition of a dorsal horn neuron responding to
noxious heating of the skin. With all 6 neurones tested, NA depressed nociceptive responses with little effect on non-nociceptive responses, so confirming previous observations.

With 5 neurones, microelectrophoretic administration of clonidine (80-90 nA, for 3-5 min) into the SG produced a prolonged (15-37 min) inhibition of excitation by both noxious heat and C fibers stimulation (Fig. 43). As shown in Fig. 42, 43., both NA and clonidine not only depressed the amplitude of these responses but also increased the latencies of excitation by the peripheral stimulus.

(b) Antagonism by Idazoxan

Table VII summarizes the effects of idazoxan on the inhibition of dorsal horn neurones by NA. Interaction between agonists and antagonist were examined by microelectrophoretic ejection both in the SG and near cell bodies, with idazoxan ejected for several minutes before the application of agonists. NA-induced inhibition was reduced or abolished with 19 out of 20 dorsal horn neurones by the ejection of idazoxan (20-50 nA for 10-24 min near cell bodies, 25-120 nA for 4-30 min in the SG) (see Table VII and Fig. 43). With all 5 neurones tested, microelectrophoretic administration of idazoxan in the SG (50-90 nA for 3-5 min) markedly reduced clonidine-induced inhibition (Fig. 43). In contrast, trimazosin (90-100 nA for 4 min), an $\alpha_1$-antagonist, had no effect on NA-induced inhibition and phentolamine (25-40 nA for 9-17 min), a non-specific antagonist of noradrenaline, produced slight reduction of NA-induced inhibition of all 3
TABLE VII NORADRENALINE-INDUCED INHIBITION OF DORSAL HORN NEURONES AND THE EFFECT OF IDAZOXAN

<table>
<thead>
<tr>
<th>RESPONSE STUDIED</th>
<th>SITE OF ADMIN.</th>
<th>EFFECT OF NORADRENALINE INHIBITION</th>
<th>N.E</th>
<th>EFFECT OF IDAZOXAN REDUCED NA-INDUCED INHIBITION</th>
<th>N.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-RESPONSE</td>
<td>SG</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CB</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NOX.HEAT RESP.</td>
<td>SG</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CB</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>SPONT.FIRING</td>
<td>SG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CB</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

SG : THE SUBSTANTIA GELATINOSA  
CB : CELL BODY  
N.E : NO EFFECT
Fig. 42. Inhibitory effect of noradrenaline (NA) on the excitation of a dorsal horn neurone evoked by noxious heat (49°). Microelectrophoetic administration of NA in the SG (50 nA) almost completely inhibited noxious heat responses (the upper record). NA-induced inhibition was reduced when idazoxan (120 nA) was ejected for 5 min before the application of NA (the middle record). The lower record shows NA-induced inhibition after ceasing ejection of idazoxan.
Fig. 43. Effect of clonidine on nociceptive responses of a dorsal horn neurone produced by noxious heat (50°) on the skin of a digital pad. Microelectrophoretic administration of clonidine (80 nA) in the substantia gelatinosa (SG) potently inhibited noxious heat responses. When idazoxan (60 nA) was ejected at the same site of the SG, this inhibition was almost abolished.
cells tested. Antagonism by idazoxan is contrasted with the lack of effect of trimazosin in Fig. 44. Fig. 45 illustrates the failure of idazoxan to reduce inhibition by GABA while abolishing inhibition by NA. This result was obtained with all 3 neurones tested.

B. Failure of Idazoxan to Reduce LC- and NKF-induced inhibition

A major problem in attempting to antagonize a supraspinal inhibition by microelectrophoretic methods is knowing where to administer an antagonist. In the present experiments idazoxan was applied both near the bodies of the neurones studied and more dorsally in the SG. In addition, the drug was also given intravenously, although the interpretation of the effects of systemic agents can be difficult.

(a) Idazoxan

In contrast to antagonism of NA-induced inhibition by administration of idazoxan, this \( \alpha_2 \) -adrenoceptor antagonist failed to block LC- and NKF-induced inhibition of dorsal horn neurones. Microelectrophoretic administration of idazoxan (25-150 nA for 3-16 min) was studied for antagonism of LC-induced inhibition of 15 neurones. On all occasions, regardless of whether the drug was ejected into the SG or more ventrally near cell bodies, no effect on LC-induced inhibition was observed. The same result was found on NKF-induced inhibition with 8 neurones. The currents and times of
Fig. 44. Effects of idazoxan and trimazosin on NA-induced inhibition. The gated C responses of a lamina V neurone by impulses in unmyelinated primary afferent fibres were inhibited, when NA (25 nA) was microelectrophoretically administered near cell body. This inhibition was reduced by ejection of idazoxan (25 nA) at the same site, but not by ejection of trimazosin (25 nA).
Fig. 45. Effect of idazoxan on NA- and GABA-induced inhibitions of spontaneous firing of a dorsal horn neurone. Ordinate indicates inhibition of spontaneous firing produced by microelectrophoretic administration of NA (24 nA) and GABA (35 nA) near cell body. Idazoxan (24 nA) only antagonized NA-induced inhibition but not GABA-induced inhibition.
ejection of idazoxan had been shown previously to reduce inhibition by noradrenaline. With two neurones inhibition by NA was alternated with that from LC stimulation. In both cases amounts of idazoxan which blocked the effect of NA had no effect on inhibition from LC stimulation (Fig. 46).

After microelectrophoresis of idazoxan, this compound was administered intravenously (0.17-0.33 mg/kg) while studying inhibition of 3 neurones. Although idazoxan produced a decrease of blood pressure, it had still no effect on LC-and NKF-induced inhibition of C responses. With another 4 neurones, idazoxan at a concentration of 10 to 50 mM was applied topically to the surface of spinal cord. The uninhibited C-responses were reduced but LC-induced inhibition was unaltered.

In addition to idazoxan, phentolamine and trimazosin were studied for possible effects on LC and NKF produced spinal inhibition. LC-induced inhibition of dorsal horn neurons was not altered by microelectrophoretic administration of phentolamine (60-120 nA, 5 min, 3 cells) or trimazosin (20 nA, 3 min, one cell) into the SG. With 2 cells, NKF-induced inhibition was unchanged regardless of the administration of phentolamine into the SG (70 nA, 5 min) or near cell bodies (40 nA, 8 min). Fig. 47 shows that there was no effect on LC-induced inhibition, when phentolamine (80 nA) and idazoxan (80 nA) were concomitantly ejected into the SG for 5 min.

(b) Other drugs

In an attempt to determine if other compounds
subserve LC- and NKF-induced inhibition, some drugs such as methysergide, bicuculline, naloxone and atropine were microelectrophoretically applied. Methysergide (100-200 nA, 5 min) administered into the SG did not modify LC-induced inhibition in 6 cells tested (Fig.48A). LC- and NKF-induced inhibition was not altered when bicuculline was administered microelectrophoretically in the SG (50 nA, 5 min in 2 cells) or near cell bodies (25 nA, 7 min in one) (Fig.48B), although basal C-responses were enhanced. Naloxone ejected into the SG (50-100 nA, 5 min) or near cell bodies (30-40 nA, 4-7 min) had no effect on LC- and NKF-induced inhibition 3 cells (Fig.45B). There was also no action of atropine on LC-induced inhibition of 2 cells when this compound was ejected into the SG (100 nA ,11 min) or near cell bodies (20 nA, 5 min).
Fig. 46. Effect of idazoxan on noradrenaline- and LC-induced inhibitions.

Both the LC stimulation (200 µA) and the ejection of NA (16 nA) near the cell body potently inhibited the gated C responses of a dorsal horn neurone produced by impulses in primary afferent C fibres of the tibial nerve. Idazoxan (20 nA) blocked NA-induced inhibition. Note that during the period of NA-induced inhibition, there was no change in LC-induced inhibition.
Fig. 47. Failure of idazoxan and phentolamine to reduce inhibition produced by stimulation of the dorsal pons. The gated C responses of a dorsal horn neurone were inhibited by stimulations of the LC (200 μA, black line) and the TAD (300 μA, broken line) (see Fig. 36). Note that LC-induced inhibition was stronger than TAD-induced inhibition. Microelectrophoetic co-administration of both phentolamine (80 nA for 19 min) and idazoxan (80 nA for 7 min) in the SG did not alter these inhibitions.
Fig. 48. No effect of microelectrophoretic administration of methysergide, idazoxan, bicuculline and naloxone on LC-induced inhibition. LC stimulation (150 µA) inhibited the gated C responses of a lamina V neurone by impulses in primary afferent C fibres following electrical stimulation of the tibial nerve. A, Co-administration of methysergide (100 nA) and idazoxan (100 nA) in the SG did not change LC-induced inhibition. B, The ejection of bicuculline (25 nA) near cell body increased basal C responses and did not reduced LC-induced inhibition. There was no effect of naloxone (30 nA) on such inhibition.
5.3. DISCUSSION

5.3.1. PAG-induced inhibition and descending pathways

A. The connection between PAG and NRM and PAG-induced inhibition

The results of the present experiments have an important bearing on current concepts of the role of medullary regions in PAG-induced inhibition of dorsal horn neurones. Since earlier anatomical studies provided no evidence for a direct projection from the midbrain PAG to lumbosacral cord levels, it was suggested that the analgesia and inhibition of spinal neurone activity produced by PAG stimulation occurs by synaptic excitation of medullary raphe neurones which project to the dorsal horn of the lumbosacral cord (Basbaum & Fields 1978).

There is considerable anatomical and physiological evidence for a PAG-medullary raphe connection. Although degeneration studies in the cat failed to find evidence for a descending pathway from the PAG to the medullary raphe (Hamilton & Skultety 1970, Hamilton 1973), the existence of the connection was clearly established in subsequent investigations employing HRP administration into the medullary raphe nuclei (particularly nucleus raphe magnus (NRM) in rats (Gallagher & Pert 1978, Senba et al 1981, Beitz 1982, Carlton et al 1983), cats (Abols & Basbaum 1981) and monkeys (Yezierski et al 1982, Chung et al 1983). Electrophysiological experiments have supported the
anatomical data. In awake rats, analgesia (noxious pinch, footshock) produced by electrical stimulation in the PAG was associated with elevations in spontaneous multiple unit activity, and decreases in noxious-evoked activity, recorded in the NRM (Oleson, Twombly & Liebeskind 1978). With single unit recording, a proportion of neurones in the NRM which were activated antidromically by spinal cord stimulation were also excited by electrical stimulation in the PAG of cats (Fields & Anderson 1978, Lovick, West & Wolstencroft 1978) and rats (Pomeroy & Behbehani 1979). Similarly, stimulation in the NRM antidromically activated neurones in the PAG in both species (Shah & Dostrovsky 1980). In behavioral studies of the rat, microinjection of glutamate into the PAG was associated not only with increased firing of NRM neurones, but with an elevated threshold for a flexion reflex to noxious heating of the skin, and this elevation was prevented by NRM lesions (Behbehani & Fields 1979). Such studies, however, have not shown directly that the NRM neurones excited from the PAG are necessarily important in producing inhibition of nociceptive responses in the dorsal horn.

B. Contribution of LRN region to PAG-induced inhibition

The present results suggest that in anaesthetized cats, the medullary raphe is relatively unimportant when compared with lateral reticular regions of the caudal medulla for the inhibition of dorsal horn neurones by stimulation of the PAG. This inhibition was reduced by about one-quarter by extensive lesions in the medullary raphe and histologically,
the lesions were considered to be functionally complete. In contrast, limited bilateral destruction in the LRN regions produced a much greater reduction of this inhibition. When this procedure followed midline lesions, the remaining inhibition was abolished. These results suggest that separate pathways to both raphe and ventrolateral medullary areas can relay the inhibitory effects of PAG stimulation to the spinal cord.

There is evidence for a substantial projection from the PAG to the ventrolateral medulla. Autoradiographic studies in the squirrel monkey have found PAG efferents projecting caudally and bilaterally to the region of the nucleus ambiguus (Jurgens & Pratt 1979, Manthy 1983), while in the cat, HRP injection into the caudal medulla near this nucleus retrogradely labelled many PAG neurones (Rose 1981, Roste, Dietrichs & Walberg 1984). Electrical stimulation in the PAG produced short-latency responses in neurones of the ventrolateral medulla, including the LRN (Rose & Sutin 1973). Neurones in the LRN region in the cat were retrogradely labelled following HRP injection into the lumbar dorsal cord (Kuyper & Maisky 1975, Basbaum & Fields 1979), indicating a spinal projection. Only recently, has the role of the LRN in control of nociceptive information been investigated. Stimulation of the LRN produced inhibition of nociceptive responses of dorsal horn neurones of the cat (Morton, Johnson & Duggan 1983), of tail flick reflexes of the rat (Gebhart & Ossipov 1986) and of jaw opening reflexes of the rabbit (Sotgiu 1985).
In the present experiments, bilateral lesions in the region of the LRN reduced tonic descending inhibition of dorsal horn neurones as well as that produced by PAG stimulation. This raises the possibility that the effects of PAG stimulation are mediated by synaptic excitation of the same lateral reticular neurones which exert tonic inhibition on dorsal horn neurones. Morton et al (1983) demonstrated that the effect of tonic inhibition could be enhanced by direct electrical stimulation in the LRN regions, and these inhibitions resemble that produced by PAG stimulation (Duggan & Morton 1983) in being selective for excitation by impulses in C fibers. In view of a study which demonstrated a considerable direct PAG-lumbar spinal projection (Manthy & Peschanski 1982), it is also possible that the lateral lesions merely interrupted the spinally-projecting axons of PAG neurones concurrently with destruction of the tonically active cells. Such a pathway, however, is not responsible for tonic inhibition, since lesions of the PAG did not reduce this inhibition (Hall et al 1982). Thus, the effects of the PAG apparently involve a connection with spinally-projecting neurones of the LRN region although further evidence is required.

The present experiments are not alone in questioning the importance of the medullary raphe for PAG-induced inhibition of nociception in the cat. The most directly comparable study used microinjections of a local anaesthetic to block the firing of neurones (and fibers) in medullary regions. Inhibition of noxious cutaneous heat-evoked responses of
dorsal horn neurones by electrical stimulation in the PAG or the mesencephalic lateral reticular formation was not affected by such injection in the NRM, but was reduced by medullary reticular injections of lidocaine (2.5 mm lateral) and abolished by injection of this local anaesthetic in both the NRM and the medullary reticular formation (Gebhart et al 1983). Although the reticular areas in these experiments were rostral and medial to those of the LRN regions considered important in the present work, both studies serve to emphasize the relative lack of importance of the medullary raphe compared with lateral reticular regions for PAG-induced inhibition of dorsal horn neurones.

Behavioral experiments in rats provide conflicting results about the importance of medullary midline structures to analgesia from PAG stimulation. Coagulation of the medial medulla blocked the increase in tail flick latency to noxious heating of the tail produced by either microinjection of L-glutamate in the PAG (Behbehani & Fields 1979) or by electrical stimulation in this area (Abbott & Melzack 1983). In this latter investigation, however, these lesions had no consistent effect on PAG-induced analgesia when using subcutaneous injection of formalin as a noxious stimulus. Prieto et al (1983), moreover, found that midline medullary coagulation had no effect on analgesia (tail flick test) produced by electrical stimulation in the PAG. These lesions did reduce the increase in tail flick latency produced by electrical stimulation ventral to the PAG. While further experiments are necessary to resolve these
discrepancies, these results question the importance of the involvement of the medullary raphe in the effects of PAG stimulation on the spinal transmission of nociceptive information.

The results of the present experiments confirm the important influence of the region of the caudal LRN in the supraspinal control of excitation of dorsal horn neurones by impulses in primary afferent C fibers. As well as having a tonic inhibitory control of nociceptive responses in the dorsal horn of anaesthetized cats, these areas are also important for the inhibition of these responses produced by electrical stimulation in the PAG.

5.3.2. Descending inhibition and noradrenaline

A. LC- and NKF-induced inhibition of dorsal horn neurones

The present study showed that electrical stimulation of the LC selectively inhibited spinal nociceptive transmission when neurones in the dorsal horn of spinal cord were excited by noxious and non-noxious stimuli. The LC-induced inhibition is similar to that produced by electrical stimulation of the PAG (Duggan & Morton 1983). As outlined in the introduction (5.1), PAG-induced analgesia in mammals may be mediated, at least partially, by inhibition of nociceptive transmission in the spinal cord. Electrical stimulation of the LC has also produced behavioural analgesia in animals (Sandberg & Segal 1978, Margalit & Segal 1979, Jones & Gebhart 1986). As with the PAG, therefore, it is
possible that LC-induced inhibition of the activity of spinal neurones may relate to behavioural analgesia produced by LC stimulation. When compared with LC stimulation, electrical stimulation of the NKF produced non-selective inhibition of both nociceptive and non-nociceptive responses of dorsal horn neurones of the spinal cord, resembling the inhibition from stimulation of the ventral tegmentum in the midbrain (Duggan & Morton 1983). Such non-selective inhibition seems unrelated to analgesia.

B. Involvement of alpha-2-adrenoceptor in NA-induced inhibition on the dorsal horn neurone

Consistent with previous observations (Headley, Duggan & Griersmith 1978), the present results indicate that the nociceptive responses of all of the dorsal horn neurones tested were selectively inhibited by microelectrophoretic NA or clonidine. This powerful noradrenaline- and clonidine-induced inhibition was markedly reduced or abolished by the α2-adrenoceptor antagonist idazoxan and slightly reduced by the non-specific antagonist phentolamine, but was unaffected by α1-adrenoceptor antagonists, regardless of whether drugs were administered into the SG or near cell bodies. The finding that idazoxan blocked the inhibition by NA, but not that by GABA, of these neurones seems to suggest a specificity in action of this compound on alpha-2 adrenoceptors in the spinal cord. This evidence further confirms the probable involvement of α2-adrenoceptors in the selective inhibition by NA of the activity of dorsal horn
neurones to noxious stimuli (Davies & Quinlan 1985, Fleetwood-walker et al 1985). However, the exact mechanism of the action of NA on dorsal horn neurones remains unknown. Some evidence suggests that an interaction between NA and SP possibly results in NA-induced inhibition. NA diminished the release of SP from cultured DRG cells (Mudge, Leeman & Fischbach 1979) and completely inhibited the noxious stimuli-induced release of SP in the dorsal horn in vivo, which was blocked by yohimbine (Kuraishi et al 1985). The co-distribution of NA and SP in the superficial dorsal horn of the spinal cord provides an anatomical substrate for such an interaction.

C. Does NA mediate LC- and NKF-induced inhibition?

A recent report revealed that LC-induced inhibition of tail flick responses in the rat was reduced by yohimbine, suggesting that this inhibition is mediated by spinal alpha-2 adrenoceptors (Jones & Gebhart 1986). The finding that idazoxan, an \( \alpha_2 \)-adrenoceptor antagonist, markedly reduced NA-induced inhibition not only supports the involvement of \( \alpha_2 \)-adrenoceptors in the selective inhibition by NA of the responses of dorsal horn neurones, but also suggests that idazoxan should reduce physiological events in the dorsal horn involving \( \alpha_2 \) receptors for NA. If NA mediates LC- and NKF-induced inhibitions, then idazoxan should block these inhibitions. In the present study, however, idazoxan did not alter inhibition by electrical stimulation in the region of medullary catecholamine-containing neurones, regardless of
whether it was administered microelectrophoretically, systemically or topically.

Several explanations can be provided for the failure of idazoxan to modify LC- and NKF-induced inhibitions of dorsal horn neurone nociceptive responses:

(a) The electrical stimulus did not activate many catecholamine-containing neurones which projected to the spinal cord, because:

(i) In the cat the majority of catecholamine-containing neurones in the LC do not project to the spinal cord (Stevens et al 1982);

(ii) The NKF in the cat is a relatively diffuse structure (Jones & Moore 1974), and it is inevitable that, even with the most precisely placed electrodes, fibers of passage as well as neurones containing or not containing catecholamines would be excited by electrical stimulation. The failure to produce inhibition by microinjection of an excitant amino acid which would excite neurones but not fibers (Goodchild, Dampney & Banaler 1982) suggests that electrical excitation of fibres of passage produced the observed inhibition from stimulation in the NKF.

(b) Inappropriate peripheral stimuli were used. Kuraishi et al (1983, 1985) reported that, in the rat, intrathecal NA had a greater effect in reducing responses to noxious mechanical stimuli than to noxious thermal stimuli. It appears that the extent of involvement of the descending noradrenergic systems in the antinociception differs with the types of noxious stimulation, and that noxious mechanical
stimulation likely is more affected. However, such noxious mechanical stimuli were not tested in the present study.

(c) The third possibility for the failure of idazoxan to reduce the inhibition which was studied is that a substance other than NA produced the observed inhibition. Consistent with this notion, the depletion of spinal NA by 5,6-DHT did not affect LC-induced inhibition in the cat (Hodge et al 1983) and the rat (Janss, Jones & Gebhart 1987). Thus, the present results may be explained by several factors, including:

(i) Large numbers of cells which do not contain NA have been found in the region of the LC of cat (Hodge 1982), and these may have been excited by electrical stimulation. The observation that microinjection of L-glutamate into the LC depressed the responses of dorsal horn neurones to impulses in primary afferent C fibers suggests that cells in the LC were excited by this amino acid. But inhibition produced by stimulation at the same site of the LC was not reduced by idazoxan. Therefore, non-adrenergic neurones in the region of the LC could be responsible for the observed inhibition;

(ii) Microelectrophoetically administered NA selectively inhibited nociceptive responses of dorsal horn neurones (Headley, Duggan & Griersmith 1978, Davies & Quinlan 1985), whereas stimulation of the NKF was non-selective. Thus, it is not unreasonable to assume an involvement of non-noradrenergic neurones in the NKF-induced inhibition;

(iii) A proportion of neurones of the LC contains neuropeptide Y (Everitt et al 1984, Yamazoe et al 1985) and
galanin (Melander, Hokfelt & Rokaeus 1986), and a plexus of fibres containing these peptides are found in the superficial dorsal horn (Ch'ng et al 1985, Skofitsch & Jacobowitz 1985). In the isolated spinal cord-tail preparation of the newborn rat, galalin (0.3-0.6 µM) depressed nociceptive reflexes produced by applying capsaicin to the tail (Yanagisawa et al 1986). The contribution of these peptides to the effects in the spinal cord of electrical stimulation of the LC is unknown but, if synergistic with those of noradrenaline, then suppression of the action of NA may be insufficient to reduce the effects of LC stimulation.

These studies indicate that considerable caution is required in relating histochemical observations to the results of physiological investigations, and thus to the function of central transmitters. Thus, whilst excitation of LC neurones apparently results in the release of NA at synapses in the dorsal horn of the spinal cord, such release alone appears not to account for the inhibition of the excitation of dorsal horn neurones by impulses in unmyelinated primary afferent fibers.
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