NEUROPEPTIDE RELEASE IN THE CAT SPINAL CORD

by

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Caelum, non animum, mutant
Qui trans mare currunt.

Horace, Epistle I

'Who rushes overseas will find
The climate changed but not his mind'

i.e. It can get fair dinkum hot here,
but I got the book finished- no worries.

Statement of Authorship

For the first half of my Ph.D., Dr.'s Arthur Duggan and Bob Morton were collaborators, involving the experiments on cutaneous stimuli evoking SP release and the tonic descending control of SP release. The second half of my degree was in collaboration with Dr. Bob Morton, on the release of CGRP, somatostatin and galanin, and the morphine and baclofen studies, in which I was a principal investigator. The studies of segmental control of substance P release, of release of dynorphin A, met-enkephalin-arg-phe and the immunohistochemistry were undertaken with minimal collaboration with other investigators.

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Abbreviations

AUS ..... area under the mean scan
CGRP ..... calcitonin gene-related peptide
CNS ..... central nervous system
cpm ..... counts per minute
CSF ..... cerebrospinal fluid
DLF ..... dorsolateral funiculus
DRG ..... dorsal root ganglion
EPSP ..... excitatory postsynaptic potential
GABA ..... gamma-aminobutyric acid
GAL ..... galanin
h ..... hours
HRP ..... horse radish peroxidase
i.p. ..... intraperitoneal
IPSP ..... inhibitory postsynaptic potential
ir ..... immunoreative
MEAP ..... met-enkephalin-arg-phe
min ..... minutes
NKA ..... neurokinin A
NKB ..... neurokinin B
PBS ..... phosphate-buffered saline
SEM ..... standard error of the mean
SP ..... substance P
SS ..... somatostatin
T ..... threshold
TEA ..... tetraethylammonium
TTX ..... tetrodotoxin

Abstract

Antibody microprobes are fine glass microelectrodes coated with specific antibodies to particular neuropeptides. They can detect nano- to micromolar concentrations of neuropeptide release in the CNS by means of an *in situ* solid phase radioimmunoassay. The precise sites of release of SP, CGRP, SS, GAL, MEAP, and dynorphin A were determined in the spinal cord of the cat following noxious thermal, mechanical and chemical stimuli to the skin, and electrical stimulation of hind limb nerves.

The tips of glass microelectrodes were coated with an amino-silane polymer and then sequentially incubated in glutaraldehyde, Protein A and an antibody directed against the neuropeptide in question. Using extracellular recording as an indication of microprobe placement and neuronal excitability, microprobes were inserted into the lower lumbar cord of barbiturate-anaesthetized cats with or without cord transection, peripheral stimulation or administration of drugs. Upon removal from the cord, microprobes were incubated in the appropriate radiolabel and then exposed to X-ray film. The resultant images were scanned by video camera and the optical density analyzed by computer. Localized deficits in binding were interpreted as sites of release of the neuropeptide *in vivo*.

A basal release of SP, SS, CGRP and GAL from the substantia gelatinosa was present in the absence of cutaneous stimuli. Noxious mechanical stimulation increased SP and CGRP release whereas noxious thermal stimulation increased release of SP, CGRP and SS. In contrast, GAL and MEAP showed no release with any of the stimuli tested. A basal release of dynorphin A was detected in lamina I and VI only with an intact spinal cord.

Since supraspinal sites are known to inhibit the activity of spinal neurones through descending fibres, the blockade of descending tracts may inhibit SP release. No change in noxiously-evoked SP release was seen with cold block or cord transection at the thoracolumbar junction, suggesting that descending inhibitory systems do not reduce the release of SP from the central terminals of nociceptors.

Activity in large diameter afferents may give rise to segmental inhibition of the transmission of impulses in small diameter fibres. Electrical stimulation of the ipsilateral tibial or sural nerve at intensities sufficient to excite only A$\alpha\beta$ or additionally Aδ primary afferent fibres did not reduce the release of SP evoked by concomitant noxious stimuli. The results suggest that segmental inhibition produced in the dorsal horn by electrical stimulation of peripheral nerves is not mediated by presynaptic inhibition of SP release from nociceptive primary afferent fibres.

Opioids have been proposed to exert their analgesic effect through a reduction of transmitter release from primary afferent terminals. However, large doses of morphine were not effective in reducing the release of SP or CGRP, suggesting that opioid drugs mediate analgesia at a postsynaptic site of action.

Baclofen reduces the release of excitatory transmitter from large diameter fibres, and has analgesic properties which may result from a similar action on small diameter fibres. While the polysynaptic excitation of dorsal horn interneurones by impulses in nociceptive afferent fibres was reduced by baclofen, no concomitant reduction in release of SP was seen in the substantia gelatinosa.

Collectively, these results suggest that the spinal transmission of nociceptive information may involve the simultaneous release and action of several neuropeptides within the dorsal horn of the spinal cord.

1. GENERAL INTRODUCTION:

Criteria for Neurotransmitter Identification in the CNS

To firmly establish that a given substance mediates transmission from primary afferent fibres to second-order spinal neurones requires a detailed examination of several aspects of the phenomenon. Several authors have laid down criteria to be fulfilled before a transmitter role may be ascribed to a candidate (Paton, 1958; Curtis, 1961; Eccles, 1964; Werman, 1966; Orrego, 1979; Salt & Hill, 1983). All authors agree on four basic criteria.

Firstly, the substance must be present in those neurones from which it is supposed to be released. For primary afferent transmitters, this criterion is inferred from statements that the substance is present in greater concentrations in the dorsal than ventral horn of the spinal cord, and the dorsal horn levels should fall after dorsal root section.

Secondly, the substance should be released into the subsynaptic space upon stimulation of the appropriate fibre type which contains the substance.

Thirdly, if the substance is present in sufficient concentrations at the post-synaptic target, the effects should be the same as the normal excitatory response to afferent stimulation.

Fourthly, the normal and induced responses should have the same pharmacological profile (order of potency) to antagonists and agonists to confirm that both effects are mediated through the same receptor or receptive system.

SUBSTANCE P

Background Information

Long before the advent of immunohistochemical procedures, SP had been identified by von Euler and Gaddum, 1931, in extracts of horse brain and intestine by its spasmogenic effect on the isolated rat jejunum and its transient hypotensive effect in atropine-pretreated rabbits (see Von Euler, 1981).

Systematic studies of the concentration of SP in the central nervous system led to the discovery of 5 - 10 times higher concentrations in dorsal than in ventral roots of the spinal cord (Pernow, 1953), leading Lembeck to propose SP as a primary afferent transmitter (Lembeck, 1953). The purification and elucidation of the amino acid sequence of SP (Chang & Leeman, 1970) led to synthesis of the undecapeptide (Tregear *et al.* 1971), enabling antibodies to be raised against the albumin-conjugated peptide for radioimmunoassay and immunohistochemical procedures (Hökfelt *et al.* 1975).

Localization within the Spinal Cord

The immunohistochemical technique has been used by many investigators to demonstrate that SP is found in the superficial laminae of the dorsal horn of several species (Hökfelt *et al.* 1975; Takahashi & Otsuka, 1975; Ljungdahl *et al.* 1978; Barber *et al.* 1979; Jessell *et al.* 1979; Gibson *et al.* 1981; Pearson *et al.* 1982; Di Giulio *et al.* 1985; Ogawa *et al.* 1985; Kawatani *et al.* 1985). The primary afferent origin of SP in the spinal cord is indicated by a decrease of irSP in the substantia gelatinosa following section of the dorsal roots, and irSP depletion by capsaicin which is neurotoxic to small sensory C fibres (Hökfelt *et al.* 1975). Substance P has been shown to be synthesized in DRG cells (Harmar *et al.* 1981) where it is transported in a colchicine-sensitive manner along the dorsal roots to be stored in central terminals. However, the majority (some 70%) is transported to the periphery where it is believed to play a role in the axon reflex and neurogenic inflammation (Pernow, 1983).

Substance P is found in about 25% of all DRG neurones and is confined to the small or type B cells of the ganglia (Ju *et al.* 1987). In the rat lumbosacral spinal cord, irSP is found in laminae I - III, Lissauer's tract and lamina X around the central canal with occasional terminals in the ventral horn (Hökfelt *et al.* 1975). A similar pattern is seen in sacral afferents in cat spinal cord (Kawatani *et al.* 1985) where irSP is found in laminae I - III, V, VII and X with

some periodicity in the concentration of staining in the rostrocaudal axis visible in parasagittal sections. Dorsoventral bundles coursing from lamina I to V along the lateral edge of the dorsal horn appear to course in a medial and dorsal direction before branching in the dorsal grey commissure in lamina X. A subcellular examination of SP immunoreactivity demonstrates its presence in large dense-core vesicles of glomerular structures thought to represent primary afferent terminals (Barber *et al.* 1979; Ribeiro-Da-Silva *et al.* 1989). In many cases this is colocalized with other neuropeptides and in some cases, biogenic amines, excitant amino acids and other putative transmitters of low molecular weight, can be found in small clear vesicles in the same synaptic terminals (Hökfelt *et al.* 1980).

The overall distribution of irSP corresponds to the termination sites of fine diameter fibres conveying nociceptive sensory information (Rethelyi *et al.* 1982; Sugiura *et al.* 1986), supporting the notion that SP is contained in, and released from these fibres.

Intraspinal Release of Substance P

It has been emphasized in several articles dealing with transmitter criteria that the release of the candidate following impulses in afferent fibres is one of the most decisive in transmitter identification (Paton, 1958; Curtis, 1961). With the availability of SP radioimmunoassay, release of irSP could be detected in perfusates of spinal cord slices following electrical stimulation of the attached dorsal rootlet (Otsuka & Konishi, 1976, 1983). Others have evoked irSP release with elevated potassium concentrations in the fluid perfusing the trigeminal nucleus caudalis (Jessell & Iversen, 1977) or the spinal cord (Sawynok *et al.* 1982; Pang & Vasko, 1986). Capsaicin has also been used as a depolarizing stimulus for SP release from slices of spinal cord (Oku *et al.* 1987). However, the change in extracellular environment inevitable in moving the preparation from *in vivo* to *in vitro* conditions is exacerbated by an elevation of

potassium ions (from 4mM to more than 50mM in some cases) to produce a non-selective and unphysiological depolarizing stimulus to all cells in the culture. In fact, the potassium-stimulated release of the contents of glial cells has been shown to be calcium-dependent, so this feature may not be proof of vesicular exocytosis (Vargas *et al.* 1977).

Further studies of SP release have been carried out *in vivo* using the techniques of push-pull cannulae (Kuraishi *et al.* 1985; Hirota *et al.* 1985), microdialysis (Brodin *et al.* 1987) and spinal catheters (Oku *et al.* 1987a; Linderoth & Brodin, 1988; Go & Yaksh, 1987). The release of SP has been detected in the intrathecal space of cat lumbar spinal cord (Go & Yaksh, 1987), where bilateral sciatic nerve stimulation at intensities exciting small diameter, slowly conducting fibres increased the release over controls by 278%. This technique, however, has the disadvantage of measurement at a site relatively remote from the area of release, the neuropeptide having to diffuse through the complicated extra-neuronal space of the neuropil, escaping enzymic degradation before appearing in the subarachnoid perfusate.

The Criterion of Physiological Identity

Receptors

The third criterion is the demonstration that the candidate interacts with the same receptors on the post-synaptic membrane to produce changes in membrane permeability to certain ions, and/or to cause the formation of intracellular second messengers.

While there are another set of criteria to be considered before a binding site is elevated to receptor status (Laduron, 1984b), several subtypes of tachykinin receptor appear to exist in various parts of the peripheral and central nervous system. In the peripheral system, the work of Iversen and others showed two distinct orders of potency for the various tachykinin agonists in *in vitro* smooth muscle preparations. In this classification, the 'P' subclass of

receptor bind SP and physalaemin preferentially and is found in smooth muscle of the guinea-pig ileum, guinea-pig bladder and rat bladder. The 'E' subclass bind the non-mammalian tachykinins, eledoisin and kassinin and are found in the rat vas deferens, rat duodenum, hamster bladder, and mouse bladder (Iversen *et al.* 1982; Jessel & Womack, 1985). Another classification by Regoli and co-workers for the mammalian neurokinins characterizes three receptor subtypes showing selectivity in three smooth muscle preparations: NK-P (dog carotid artery), NK-A(rabbit pulmonary artery) and NK-B (rat portal vein), each with a unique rank order of potency with the most potent ligand giving the name to the receptor type (Regoli *et al.*1987).

There appears to be less evidence for distinct subtypes of neurokinin receptors in the central nervous system. Physalaemin and eledoisin are several times more potent than SP in depolarizing rat spinal cord motoneurones *in vitro* suggesting yet another subtype of receptor (Konishi & Otsuka, 1974). However, the various potencies of agonists in these preparations may reflect different pharmacokinetic properties, such as access to receptors and differential degradation in various tissues. The approach taken to elucidate types and distribution of CNS receptors has been one of autoradiographic localization of binding of the radioiodinated SP agonists to sections of brain tissue (Quirion & Dam, 1986), where it appears there are two separate types of receptors, NKA and SP. In the spinal cord (Charlton & Helke, 1985b; Charlton & Helke, 1986; Helke *et al.*1986), SP receptors appear to be located on the postsynaptic membrane since destruction of sensory afferents with botanical toxins (ricin and capsaicin) increases the density of binding sites akin to a denervation-supersensitivity phenomenon. With the advent of new antagonists (*vide infra*), it may be more informative to characterise the receptors by displacement of a radiolabeled antagonist by the various agonists. Despite details concerning what type of tachykinin receptor is present, there appears to be good evidence for SP receptors in the superficial layers of the dorsal horn.

Postsynaptic Membrane Actions

Many different *in vivo* and *in vitro* preparations have been used to demonstrate the excitatory action of SP on neurones of the spinal cord. The microelectrophoretic administration of SP onto dorsal horn neurones produces a slow onset (30 - 60 s) and long lasting (several minutes) increase in firing rate of a subpopulation of cells (Henry, 1983; Murase & Randic, 1984). This delayed onset may be due to a slow rate of ejection from micropipettes by electric current or slow diffusion to its site of action, and the long-lasting action may be due to an absence of a re-uptake mechanism, a conversion to active metabolites, and/or slow degradation (Piercey *et al.* 1985). In other studies, it was shown that SP-sensitive dorsal horn neurones were specifically excited by noxious, but not non-noxious chemical stimuli (Piercy *et al.* 1980).

In rat spinal cord slices, the presence of micromolar concentrations of SP in the perfusion medium produced a slow depolarization and increase in excitability of dorsal horn interneurones which was similar to the responses induced by dorsal root stimulation at C fibre threshold (Urban & Randic, 1984; Akagi *et al.* 1987).

The shortcoming in these experiments is the inability to demonstrate that the neurone under study is monosynaptically excited by the primary afferent C fibre which releases SP. The asynchronous input of many small diameter C fibres of varying conduction velocity precludes an identification of monosynaptic input. In the absence of this information, it cannot be ruled out that an interneurone containing SP or some other excitatory transmitter is presynaptic to the recording site.

Ionic Channels Activated by SP

There is some controversy as to the particular ionic conductances that are responsible for the slow depolarizing response of SP on spinal neurones.

The SP-induced inhibition of the M-current (muscarinic-sensitive potassium current) in cultured mouse spinal cord neurones (Nowak & Macdonald, 1982) and the inward rectifying potassium current in dissociated neurones (Stanfield *et al.* 1985) was not sufficient to account for the slow depolarizing response observed in studies of immature rat spinal cord slices *in vitro* (Murase *et al.* 1986). Instead, many neurones were found to have slow calcium-sensitive currents which were enhanced in the presence of SP (Murase *et al.* 1986). A more recent voltage-clamp study of neurones in the rat spinal cord slice again ruled out any changes in the inward rectifying potassium current, reporting only a small reduction in the M-current and confirming the SP-induced enhancement of a slow persistent calcium current, which may have been secondary to non-specific increases in membrane permeability to sodium and potassium (Randic & Murase, 1988b).

Antagonists of substance P

The development of antagonists of SP will allow a better characterization of receptor types and will help determine whether SP is a transmitter of primary afferent neurones, but the real achievement may be the production of superior analgesics for the treatment of pain of nociceptive afferent origin.

The first antagonists were made by the substitution of the dextrorotatory enantiomers of amino acids into the sequence of the undecapeptide, which hinders enzymic degradation. The essential structural features of the SP analogues which confer antagonist properties are; at least two D-trp residues at the seventh and ninth positions, a C-terminal methionine-amide group replaced with either leucine-amide or phenylalanine-amide to increase the hydrophobicity of the peptide, and the substitution of L-arginine to improve the aqueous solubility (Michelot *et al.* 1988).

The first antagonist synthesized by Rosell & Folker's group, D-pro^{2}, D-phe^{7}, D-trp^{9} - SP was shown to antagonize a caudally-directed biting and scratching ('nociceptive') behaviour after intrathecal administration (2μg/rat)but produced paralysis at marginally higher doses (4μg/rat in (Akerman *et al.* 1982), also see (Piercey *et al.* 1985). The slow excitatory response elicited by micromolar concentrations of SP in rat spinal cord slices was reduced by the presence of the SP antagonist, spantide (D-arg^{1}, D-pro^{2}, D-trp^{7,9}, leu^{11}-SP) and recovery was observed on wash-out (Urban & Randic, 1984; Randic *et al.* 1988a) While some workers have reported that this compound has local anaesthetic (Post *et al.* 1985; Post *et al.* 1987) or even neurotoxic effects (Post & Paulsson, 1985) when placed into the subarachnoid space, these effects depend on the dose given, and these tests may not provide the sensitivity to detect a therapeutic 'window'.

The tachykinin antagonist D-pro^{2}, D-trp^{7,9} - SP was selective in its reduction of the depolarizing responses of eledoisin and kassinin, but not of SP or physalaemin, on the ventral root potential of the isolated spinal cord of the newborn rat (Briggs *et al.* 1982). The ventral root depolarizations induced by capsaicin were also reduced by the antagonist, suggesting that the capsaicin response is mediated through tachykinin 'E' receptors on the postsynaptic membrane. Kassinin and eledoisin not found in mammals, so it seems incongruent that this analogue does not block the effect of the naturally occuring tachykinin.

The intrathecal administration of the SP analogue, D-trp^{7,9,10} was tested on the reaction time in a tail flick response to a noxious thermal stimulus (Couture *et al.* 1985). The decrease in reaction time induced by physalaemin was reversed by the analogue, but the basal reaction time was not affected. The lack of antagonism of the endogenous nociceptive signal in the face of blockade of an exogenously administered non-mammalian agonist suggests some physical inactivation of the two peptides when administered together. This type of

experiment seems to be confounded by the fact that the analogues induce flaccid paralysis after intrathecal administration, casting doubt on the validity of this 'analgesic' test. For example, the motor dysfunction induced by one SP antagonist may be due to ischemic trauma following the constriction of blood vessels in the ventral horn (Barber *et al.* 1987). It seems that these studies of SP analogues have not satisfactorily demonstrated a pharmacological antagonism of the effects of SP released in the spinal cord.

The development of non-peptide antagonists has the advantage of a possible systemic route of administration, a desirable step forward in the development of analgesics of therapeutic value. Piperazinone derivatives of the C-terminal hexapeptide of SP have shown a behavioural antagonism of the SP-induced biting and scratching responses following intrathecal administration, but they are not active following systemic administration (Piercey *et al.* 1986).

Just as collection of SP from intrathecal catheters is unable to locate the site of release of SP, the administration of SP antagonists via spinal catheters will yield no information on the site of action of these compounds. Pial nerves, blood vessels and glia are exposed to the same (or probably higher) concentration as the postsynaptic membrane of neurones receiving a C fibre input and may produce effects which complicate the interpretation. More specific antagonists are needed which should be administered in the substantia gelatinosa by microelectrophoresis, if possible, so that the question of SP in nociceptive transmission may be more confidently addressed.

Summary Remarks

It appears that SP fulfils the criteria of localization and release, but the exact site of release has not yet been convincingly demonstrated. The physiological identity criterion has been fulfilled in a general way , but more evidence for receptor subtypes is needed in the CNS as has been shown for peripheral tissues. There is still controversy over the precise ionic mechanisms

involved in SP-induced depolarizations. Finally, more specific and selective antagonists are needed which may be administered in the vicinity of the first nociceptive synapse, to address the criterion of pharmacological identity.

CALCITONIN GENE-RELATED PEPTIDE

Background Information

Calcitonin gene-related peptide (CGRP) was discovered using techniques of molecular biology. Calcitonin is secreted from the C-cells of the thyroid gland and decreases the level of calcium in the blood by promoting bone formation and by other means which physiologically antagonize the effects of parathyroid hormone. The mRNA for the calcitonin gene was found to encode extra peptide fragments which are produced in other tissues. Whereas the thyroid, as expected, produces mainly mRNA for calcitonin, the central nervous system expresses mRNA for another peptide sequence which is translated into a 16 kilodalton precursor to yield α- or β-calcitonin gene-related peptide, which differ by only one amino acid. This novel approach to peptide discovery is in contrast to SP and SS, where biological activity in bioassay systems led to the isolation of the active component.

Spinal localization of irCGRP

In several species including the cat, large numbers of CGRP-containing DRG neurones have been identified using polyclonal antibodies raised with rat CGRP conjugated with adjuvants (Gibson *et al.* 1984; Molander *et al.* 1987). Within these neurones, CGRP-like immunoreactivity appears at the axon terminals of unmyelinated and finely myelinated primary afferent fibres (Carlton *et al.* 1987; McNeill *et al.* 1988) and is located within large dense cored vesicles. Both in DRG neurones and axon terminals in the CNS, there appears to be an extensive colocalization of irCGRP with irSP (Lee *et al.* 1985ab; Skofitsch & Jacobowitz, 1985d; Molander *et al.* 1987). This colocalization

appears to occur even to the extent that individual vesicles contain both irSP and irCGRP in peripheral nerve endings (Gulbenkian *et al.* 1986; Alvarez *et al.* 1988) and DRG neurones (Merighi *et al.* 1988), and by the inference of Dale's principle (Eccles, 1986), in the central terminals of primary afferents in the spinal cord. In the guinea-pig, a double immunogold technique was used to demonstrate irCGRP and irSP in the same vesicles in peripheral nerve fibres, the DRG and its brainstem homologue, the trigeminal ganglion (Gulbenkian *et al.* 1986).

CGRP appears to be the most ubiquitous peptide found in sensory ganglia at this time. In the rat, 50% of all lumbar and cervical DRG neurones, and 70% of the small and medium diameter neurones contain irCGRP (Lee *et al.* 1985b). In the cat, the ratio of irSP to irCGRP DRG neurones was found to be 1 : 2.2 - 2.7 (Gibson *et al.* 1984; Cameron *et al.* 1988) whereas a 1 : 1 ratio was found in the guinea-pig (Gibbins *et al.* 1987). This discrepancy may be methodological, since the guinea-pig ganglia were excised whole and cultured in a colchicine-containing medium before fixation, giving a longer period of time for SP levels to increase. In the rat vagus nerve, irCGRP was found in about 5% of myelinated fibres and about 50% of unmyelinated fibres, located in large, dense-cored vesicles but not small, clear vesicles (Kakudo *et al.* 1988).

The near-complete reduction in irCGRP following removal of the synthetic source in the DRG by sectioning the dorsal roots, suggests that descending fibres and neurones intrinsic to the dorsal horn contribute little to the irCGRP in the dorsal horn (Chung *et al.* 1988). This has led some authors to regard CGRP as a 'sensory marker'. For example, the short ascending portion of small diameter primary afferent fibres has been located in the dorsolateral funiculus as opposed to the tract of Lissauer (McNeill *et al.* 1988), based on the presence of irCGRP in the former structure. However, irCGRP is also found in motoneurones in the ventral horn (Skofitsch & Jacobowitz, 1985a), where it may play some efferent role at Ach receptors of the neuromuscular junction

(see Kruger *et al.*, 1988). Others have proposed a specific role for CGRP in nociception based on an increased incidence of irCGRP synaptic contacts with dynorphin A profiles in the dorsal horn of arthritic rats (Takahashi & Traub, 1988), but further evidence of nociceptive-specific release of CGRP must be demonstrated.

CGRP Release

Compared to the abundance of studies demonstrating immunohistochemical localization of CGRP in fixed tissues, relatively little has been published on the release of this putative neurotransmitter from neurones known to contain this neuropeptide.

In rat spinal cord slices, 60 mM potassium or 10μM capsaicin has evoked the release of irCGRP, irSP and irNKA into the artificial CSF (Gamse *et al.* 1981). The basal and potassium-evoked release of CGRP and SP were comparable in magnitude but the capsaicin-evoked release of CGRP was greater than that of SP, perhaps reflecting a somewhat more selective action of capsaicin. Cultured rat trigeminal ganglion cells were shown to exude irCGRP upon depolarization with 59mM potassium in a calcium-dependent fashion (Mason *et al.* 1984). However, the 60 min period of exposure to high potassium led to a depletion of 30% of the cellular stores of CGRP, which was reproducible only after a further 18 - 24 h in culture. Chemical stimuli such as capsaicin and bradykinin have also evoked release of irCGRP from peripheral sensory terminals in preparations of guinea-pig heart (Geppetti *et al.* 1988) and urinary bladder (Maggi *et al.* 1988).

Since no evidence of *in vivo* release of irCGRP has been forthcoming, the studies presented in this thesis were undertaken to demonstrate reproducible release of small amounts of CGRP upon depolarization of nerve terminals by action potentials in primary afferent fibres.

The Criterion of Physiological Identity

Receptors

Specific, saturable binding sites have been described in homogenates of spinal cord and brain of the rat (Goltzman & Mitchell, 1985), but this is of little use in ultrastructural localization of putative receptors. In rat and human CNS, autoradiography of spinal cord and brain sections has shown a dense binding of radioiodinated CGRP in the substantia gelatinosa of the human trigeminal nucleus caudalis (Inagaki *et al.* 1986). However, the cervical region of the spinal cord showed no distinct binding in the substantia gelatinosa but fairly even binding over the gray matter and dorsolateral funiculcus, questioning whether an appropriate exposure time was employed for these sections. In addition, the presence of exposed silver grains on the autoradiographic image corresponding to the DLF is rather puzzling, considering the localisation of irCGRP within fibres of passage in this area (McNeill *et al.* 1988). Perhaps, some other macromolecule (ion channel, transport protein) within small diameter fibres containing CGRP is giving a false impression of 'receptor' localization (Laduron, 1984b), because this study does not show the expected distribution of CGRP binding sites in the spinal cord.

In contrast, Skofitsch & Jacobowitz (1985) have shown a selective distribution of ^{125}I-tyr^{0}-CGRP binding sites in the dorsal horn and regions surrounding the central canal. Within the dorsal horn, the superficial layer appeared to have slightly greater binding of the tracer, a finding which is consistent with the localization of irCGRP.

Membrane actions

The *in vitro* effects of CGRP on the membranes of dorsal horn interneurones has been studied in the immature rat spinal cord slice which has been cut to preserve the dorsal root ganglia of that segment (Ryu *et al.* 1988). A micromolar solution of CGRP produced a slow, reversible depolarization in one-third of all cells examined, whereas a few cells showed an initial

hyperpolarization followed by a prolonged depolarization. Since the CGRP - induced depolarization was unaffected by tetrodotoxin (sodium channel blocker) and tetraethylammonium (potassium channel blocker), it was suggested that enhanced calcium entry may underlie the slow EPSP. In a parasympathetic ganglia of the cat urinary bladder, an interaction of CGRP with calcium has also been suggested by the enhancement of calcium-dependent potentials in the presence of calcitonin and CGRP (Nohmi *et al.* 1986). Furthermore, synaptosomes prepared from rat dorsal horn show a nifedipine-reversible enhancement of $^{45}Ca^{2+}$ entry in the presence of nano- to micromolar concentrations of CGRP (Oku *et al.* 1988). It may not be fortuitous that two peptide products of the same calcitonin gene are both involved in mobilizing stores of calcium.

A similar membrane effect of CGRP occurs *in vivo*. In cat lumbar dorsal horn neurones, the microelectrophoretic application of CGRP produced an excitation of slow onset (30 s to 3 min) and long duration (10 min)(Miletic & Tan, 1988). Although 10 out of 18 cells examined for their response properties showed excitation, none of the 4 nociceptive-specific type of cell was excited by application of CGRP, and there was no evidence for monosynaptic excitation. The observation of vesicular colocalization begs the question of whether these cells would respond to simultaneous microelectrophoretic administration of SP and CGRP, as is likely the case under natural conditions. Interactions of these two peptides have been described both *in vitro* and *in vivo*. CGRP retards the degradation of SP (Le Greves *et al.* 1985) and can potentiate the release of SP from the dorsal horn (Oku *et al.* 1987). An interesting microelectrophoretic study would be to examine the effect of CGRP on the nociceptive neurone while it is being excited by endogenously released SP.

In contrast to the unimpressive membrane action reported in the spinal cord, a fast excitatory response has been reported following pressure ejection of CGRP in the vicinity of guinea-pig coeliac ganglion cells. This fast response

was not sensitive to TTX but was attenuated by d-tubocurarine and abolished by a sodium-free external medium, suggesting to these authors that CGRP acts on a TTX-insensitive sodium channel (Dun & Mo, 1989). The slow depolarizing response seen in the above-mentioned *in vivo* study was also observed in this preparation but was not affected in low calcium - high magnesium ion solution.

In myenteric neurones another mechanism has been proposed to give rise to the slow depolarizing responses of the plasma membrane. The application of CGRP inactivated a potassium conductance which itself was calcium-activated. While it has been shown that irCGRP fibres in the coeliac ganglion may be peripheral branches of primary afferent fibres with their cell body in the DRG (Gibbins *et al.* 1985), it is not known if a fast excitatory response is a central action of CGRP released in the spinal dorsal horn.

Studies by Wiesenfeld-Hallin *et al.* (1984) have revealed a functional interaction of CGRP with two other neuropeptides, SP and SS, which are colocalized to a greater and lesser degree, respectively, in the sensory system (Skofitsch & Jacobowitz, 1985d). The intrathecal administration of SP alone produced a 'nociceptive' behaviour of caudally directed biting and scatching which lasted several minutes. Concomitant administration of SP (10 μg) and CGRP (20μg) produced a response of greater frequency and duration (30 min), whereas CGRP (20 μg) by itself had little effect. The physiological identity is apparent from a similar pattern with nerve stimulation. The increase in excitability of the flexor reflex by a sural C fibre conditioning stimulus was enhanced by intrathecal CGRP and further increased by CGRP and SP.

As discussed below, the nociceptive behaviour and flexor reflex produced by somatostatin is also potentiated by intrathecal (0.2 - 2.0 μg) CGRP (Wiesenfeld-Hallin, 1986a). It appears, then, that the physiological mimicry of nociceptive behaviour and withdrawal reflexes by CGRP is only present in the

context of an organized response involving other transmitter candiates, such as SP and SS.

Antagonist of CGRP

At present there are no CGRP analogues that are effective as antagonists. The intrathecal infusion of antibodies to CGRP has been reported to increase the nociceptive threshold for mechanical stimulation of the hind paw in both normal and arthritic rats, suggesting that CGRP facilitates nociceptive function (Kuraishi *et al.* 1988).

SOMATOSTATIN

Localisation within the spinal cord

Immunoreactive somatostatin has been found in cat DRG cells (Leah *et al.* 1985b; Kawatani *et al.* 1986) and in the superficial laminae of the cat dorsal horn (Krukoff *et al.* 1986; Tessler *et al.* 1986) and other species (Ho & Berelowitz, 1984; Price, 1985). In the spinal cord SS-immunoreactivity is confined to neural elements, fibres and cell bodies in lamina I, II and III. Less than 10% of rat DRG neurones were found to contain somatostatin compared with the 15-20% observed for SP (Molander *et al.* 1987). Other estimates of peptide immunoreactivities in rat DRG neurones have been higher with about 20% for SS and about 30% for SP (Ju *et al.* 1987). In initial studies in rats, these DRG immunoreactivities appeared to have mutually exclusive neuronal distributions, suggesting a functional differentiation between SP- and SS-containing neurones (Hökfelt *et al.* 1976). Subsequent studies, however, have shown occasional (7.8%) coexistence of irSP and irSS in rat DRG neurones (Molander *et al.* 1987). In the cat, 5 - 7% of DRG neurones contain irSS and a high proportion (72.7%) of these also contain irSP, but a low proportion (13.6%) also contain irCGRP (Cameron *et al.* 1988).

The relative contribution of intrinsic spinal neurones to the overall

content of irSS in the dorsal horn is somewhat greater than SP since a 40% in irSS follows dorsal rhizotomy (Tessler *et al.* 1986) and 80% decrease is seen with SP (see p. 3). In contrast, capsaicin treatment of the sciatic nerve of the rat produced a 48% depletion in somatostatin and only a 27% depletion of SP, suggesting that a sub-population of SP-containing cells are resistant to capsaicin (Wall *et al.* 1981).

There appears to be a descending component of irSS in the spinal cord which closely matches the pathway of the dorsal reticulospinal tract, originating in cell bodies of the nucleus gigantocellularis and nucleus raphé magnus extending down the central medullary tegmental path to terminate in the dorsal horn (Bowker & Abbott, 1988). This suggests that irSS release may be of efferent as opposed to afferent origin in the spinal cord (Shimada *et al.* 1985; Schrader, 1985; Millhorn *et al.* 1987). It is tempting to speculate that somatostatin is involved in inhibition from supraspinal sites, since the perikarya of irSS fibres and the location of tracts are consistent with anatomical sites implicated in descending inhibition.

Release of irSS from spinal cord

Experiments utilizing a push-pull cannula have detected release of irSS from the rabbit lumbar dorsal horn with noxious cutaneous thermal stimulation of the hind limb (Kuraishi *et al.* 1985). Interestingly, it was found that noxious mechanical stimulation did not evoke release of irSS, suggesting that some functional characteristics may be indicated by the neuropeptide content of the neurones.

In vitro preparations of rat spinal cord have shown that potassium (60 mM) (Sheppard *et al.* 1979) or capsaicin (Gamse *et al.* 1981) results in the appearance of irSS in the perfusion fluid, but the relative contribution of intrinsic interneurones, and the terminal fragments of neurones of descending and primary afferent origin to the levels of irSS is impossible to determine.

Apparently, no studies have utilized i.t. catheters for collection of irSS from spinal perfusates.

Physiological Identity Criteria

Somatostatin binding sites

In the rat, analogues of SS_{1-14} and SS_{1-28} were used as autoradiographic tracers to localize binding sites in the substantia gelatinosa of the spinal cord and trigeminal nucleus caudalis (Uhl *et al.* 1985). The substantia gelatinosa of postmortem human spinal cord has also been shown to be dense with somatostatin binding sites (Reubi *et al.* 1986; Charnay *et al.* 1987, 1988). There appear to be no reports of the cat spinal cord showing somatostatin binding sites.

Ionic Channels

Randic & Miletic (1978) have shown predominantly inhibitory effects of microelectrophoretic SS administered to the spinal cord isolated from immature rats. An increase in action potential amplitude was associated with a depression of firing frequency of neurones, suggestive of a hyperpolarization. A few nociceptive units were found to be weakly excited in lamina VII of the dorsal horn. Further work by Murase *et al.* (1982) showed that in the majority of cells studied (15/18), micromolar concentrations of somatostatin produced a dose-dependent and reversible hyperpolarization associated with a small decrease in membrane input resistance. This membrane effect of SS is similar to that seen in neurones of the gastrointestinal tract (Mihara *et al.* 1987).

The membranes of submucous plexus neurones were studied to determine if release of somatostatin from efferent sympathetic nerves could result in transmitter-like actions (Mihara *et al.* 1987). More than 90% of neurones were hyperpolarized by somatostatin with an increase in inward rectification of potassium ions. The effects of somatostatin were sensitive to

guanine nucleotides, suggesting an involvement of a G-protein in the coupling between a presumed receptor site and the ion channel. A similar inhibitory membrane action may exist for somatostatin released in the spinal cord from afferent fibres with cell bodies in the DRG, but this contradicts the widely held belief that all primary afferent transmitters are excitatory.

A role for somatostatin in nociceptive transmission is supported by the physiological identity of nociceptive behaviour following intrathecal administration to decerebrate, spinalized rats (Wiesenfeld-Hallin, 1985). A caudally-directed scratching and biting behaviour, seen after small doses of somatostatin, is suggestive of pain perception. This result is somewhat surprising considering the analgesic action of SS in humans after intrathecal administration (Chrubasik *et al.* 1984, 1985), and the inhibitory effects on dorsal horn neurones just described. A high dose of intrathecal SS may lead to ischaemic tissue damage from constriction of blood vessels (Mollenholt *et al.* 1988), and activation of sensory afferents in the vasculature or meninges may give rise to a nociceptive input not accounted for in these studies. Further physiological similarities are evident from electrophysiological recording of the nociceptive flexor reflex, where both intrathecal somatostatin and C fibre conditioning stimuli increase the excitability of the hamstring flexor reflex. The increase in excitability of the nociceptive reflex was specific to a noxious heat stimulus but did not occur with a noxious mechanical stimulus (Wiesenfeld-Hallin, 1986a,b).

Somatostatin Antagonists

A blockade of the effects of somatostatin in the spinal cord with analogues of somatostatin has not been attempted to date. In the work of Mihara *et al.*(1987) the inward movement of potassium ions during rectification of the membrane potential was not blocked by the somatostatin analogue, cyclo-aminoheptanoyl-Phe,D-trp,lys,(benzyl) - SS, which has been reported

effective in inhibiting growth hormone release from cultured hippocampal cells and stimulating growth in rats (Spencer & Hallet, 1985).

GALANIN

Background Information

After translation from mRNA many peptides are chemically modified at amino acid residues, which changes their biological activity. In mammals, the N-amidation of the C terminal of many peptides occurs only in neuroactive or hormonally-active peptides. The isolation of peptides containing an N-amidated C terminal structure has produced some new biologically active peptides, such as neuropeptide Y, peptide YY, and neuropeptide K. This approach also led to the discovery of galanin in the pig intestine (Tatemoto *et al.* 1983); the peptide deriving its name from **gl**ycine and **al**anine present at the N- and C-terminals of the peptide, respectively (Rökaeus, 1987).

Spinal Localization

The production of antibodies against synthetic galanin enabled immunostaining in the CNS, revealing a differential pattern of staining in various somatosensory regions (Skofitsch & Jacobowitz, 1985c,e, 1986). IrGAL has been measured by radioimmunoassay and immunohistochemistry in the spinal cord of pig and rat (Ch'ng *et al.* 1985; Skofitsch & Jacobowitz, 1985c,e; Melander *et al.* 1986; Ju *et al.* 1987; Bauer *et al.* 1988). In the rat, cell bodies containing irGAL have been observed in the superficial layers of the dorsal horn throughout the spinal cord, and additionally, in the vicinity of the central canal at the lumbosacral level (Skofitsch & Jacobowitz, 1985c; Melander *et al.* 1986). The ventral horn of the rat spinal cord has been shown to contain a few immunoreactive motoneurones. A population of small DRG neurones were found to be galanin-ir, which disappeared after capsaicin treatment

indicating the presence in primary sensory C fibres (Skofitsch & Jacobowitz, 1985e).

Release of Galanin

No studies have appeared on galanin release *in vivo*.

Physiological Identity Criterion

Specific binding sites for radioiodinated galanin have shown agreement with the localization of irGAL in the sensory trigeminal region and dorsal laminae of the spinal cord (Skofitsch *et al.* 1986). A class of single, high affinity galanin receptor sites in rat brain membranes was isolated using the radioiodinated peptide as an affinity label and the high MW protein was found to be saturable, reversible and specific to the peptide (Servin *et al.* 1987). No reports on the effects of galanin on ionic channels or second messenger systems have been found at this time.

Intrathecal galanin administration appears to inhibit nociceptive reflexes (Post *et al.* 1988; Cridland & Henry, 1988), but in view of the effects of galanin on motoneurones discussed below, a paralytic effect may be responsible for the increases in response latency in tail-flick and hot plate tests observed in the mouse (Post *et al.* 1988). In direct opposition to these 'analgesic' effects, intrathecal galanin has been shown to be hyperalgesic in behavioural tests of mechanical nociception (Cridland & Henry, 1988). Galanin lowered the threshold to a tail pinch stimulus such that a previously innocuous level of stimulation induced vocalization in these animals.

In the hemisected spinal cord of the newborn rat, galanin depressed a monosynaptic ventral root reflex elicited by dorsal root stimulation and reversed the increase in the reflex produced by bicuculline, strychnine or naloxone (Yanagisawa *et al.* 1986). In the same report, the administration of capsaicin to the isolated spinal cord and tail preparation produced a nociceptive

reflex recorded in the ventral root, which was reversibly inhibited by galanin in concentrations of 0.3 - 0.6 μM. While these authors interpret these results in favour of galanin as an inhibtory transmitter of group Ia primary afferent fibres, the immunohistochemical presence in this region of the ventral horn is not consistently observed (Ch'ng *et al.* 1985; Skofitsch & Jacobowitz, 1985c; Melander *et al.* 1986), and it is generally believed that large DRG cells do not have peptide-containing vesicles, leaving the criterion of presence insufficiently demonstrated.

OPIOID PEPTIDES

There is little evidence to date that opioid peptides are candidates for primary afferent transmitters, and their predominantly inhibitory effect in the spinal cord would seem to preclude this. For this reason, the physiological and pharmacological criteria used for the previous peptides has not been strictly followed. Instead, a general background of information has been given for the two opioid peptides investigated in this work.

MET-ENKEPHALIN-ARG-PHE

Background, presence and localization

The discovery of the mRNA sequence of the proenkephalin precursor revealed that extended versions of met^5- and leu^5- enkephalin also exist in the genetic code for enkephalin peptides. Of the 6 copies of met-enkephalin found in the sequence of pro-enkephalin, two of these have C-terminal extensions, met-enkephalin-arg^6-gly^7-leu^8 and met-enkephalin-arg^6-phe^7 (irMEAP) and there are also larger fragments (Comb *et al.* 1982; Gubler *et al.* 1982; Noda *et al.* 1982). The presence of irMEAP was determined in rat spinal cord by radioimmunoassay of acidic extracts of individual spinal cord segments (Majane *et al.* 1983). The lumbar and sacral segments were found to contain more irMEAP than cervical and thoracic segments and the dorsal gray matter

contained about twice as much as the ventral gray matter. In the rat, irMEAP nerve fibres were detected at the sixth cervical to sixth thoracic level in the dorsal horn, ventral horn, and the intermediolateral column. However, no irMEAP was detected in the spinal ganglia at this level.

Release of irMEAP

Tang *et al.* 1983 have detected the release of irMEAP from the superfusates of the rat spinal cord, but only in the presence of several peptidase inhibitors suggesting that it is degraded very quickly, and the release increased after intrathecal administration of SP. Release of irMEAP has been demonstrated from the cat superior cervical ganglion *in vitro* where it may modulate the release of other transmitters (Araujo & Collier, 1987). For example, the release of acetylcholine from the ganglion into the perfusate was depressed by MEAP in a naloxone-reversible manner, suggesting that opioid receptors may be involved in presynaptic inhibition of acetylcholine release from the ganglia. MEAP seems to have an opiate-like effect in the spinal cord since intrathecal administration produces analgesia (Inturrissi *et al.* 1980).

Anitbodies are available with a much higher sensitivity of detection of this heptapeptide form of enkephalin than the pentapeptides (Terenius, pers. comm.), enabling a sensitive and specific antibody microprobe assay of the release in the cat spinal cord.

DYNORPHIN A

Spinal Localization

The presence of dynorphin-related peptides in laminae I-II and V-VI of the spinal cord of the rat (Botticelli *et al.* 1981; Cox *et al.* 1985; Sasek & Elde, 1986; Weihe *et al.* 1988, Miller & Seybold, 1987, 1989) and cat (Basbaum *et al.* 1986; Miller & Seybold, 1987, 1989) has been demonstrated by immunohistochemical techniques. Although most ir-dynorphin occurs in spinal

interneurones, a proportion of this derives from primary afferent fibres. In the guinea pig, ir-dynorphin A (1-13) is present in DRG cells of lumbosacral origin (Gibbins *et al.* 1987). Both dynorphin A and dynorphin B immunoreactivities have been found in dissociated cell cultures of murine DRG and spinal neurones (Sweetnam *et al.* 1986). Following unilateral deafferentation (L5 to S3) in the cat, dynorphin B immunoreactivity was decreased in sacral but not lumbar segments, suggesting that most ir-dynorphin B at lumbar levels is associated with interneurones (Basbaum *et al.* 1986). In agreement with the dynorphin immunoreactivity, mRNA encoding prodynorphin-related peptides can be localized to lamina I-II and V-VI of the spinal cord (Höllt *et al.* 1987; Ruda *et al.* 1988) of rats with experimentally-induced peripheral inflammation.

Release from spinal cord

Several studies have attempted to measure dynorphin release in the mammalian spinal cord. Perfusion of artificial CSF in the spinal arachnoid space, coupled with radioreceptor assay (Nyberg *et al.* 1983; Yaksh *et al.* 1983) or radioimmunoassay (Xie *et al.* 1986; Fei *et al.* 1986), has shown release of a peptide with chromatographic properties similar to dynorphin A following electrical stimulation of peripheral nerves in the rat, rabbit and cat. The work of Han's group suggests that dynorphin A is released in high frequency electroacupuncture analgesia. In the rabbit, such analgesia can be blocked by the intrathecal injection of anti-dynorphin antibodies (Han & Xie, 1984) and in the rat, high frequency electroacupuncture increased the ir-dynorphin A content of lumbar spinal superfusates (Fei *et al.* 1986).

Dynorphin A has also been associated with the production of ischaemia and trauma following spinal cord injury (Faden *et al.* 1985a; Faden, 1986). An increase in spinal dynorphin A levels has followed traumatic injury to the thoracic cord (T10) sufficient to cause paraplegia (Cox *et al.* 1985; Faden *et*

al. 1985b). Increased kappa opioid binding was associated with the spinal injury (Krumins & Faden, 1986) and a selective kappa opioid receptor antagonist enhanced the recovery process (Hall *et al.* 1987).

2. STATEMENT OF OBJECTIVES

Several studies to date have measured the spinal release of various peptides. It has become increasingly clear that many of the *in vitro* studies provide evidence that is contradictory to work obtain in whole animal experiments, and in the final analysis the emphasis must be placed on *in vivo* experimentation. The antibody microprobe technique developed in this laboratory provides many advantages over other techniques employed for *in vivo* studies of neuropeptide release. This justifies a re-examination of several experiments with SP release to validate the current technique and to provide a framework for further investigations of novel sensory peptides.

The objective of this study is to survey a number of neuropeptides occurring in the mammalian spinal cord to identify candidates that may be involved in sensory physiology. Particular emphasis is placed upon the demonstration of stimulus-dependent and nociceptive-specific release of the neuropeptide in question. One further goal of this investigation is to undertake a more detailed examination of the physiology and pharmacology of the stronger candidates of primary afferent transmission.

3. METHODS

Animal Preparation

Experiments were performed on cats (2.0 - 4.0 kg) anaesthetized with pentobarbitone sodium (20 - 35 mg/kg intraperitoneal) and the left cephalic vein, a carotid artery and the trachea were cannulated. The spinal cord was exposed by laminectomy following a midline skin incision and removal of the erector spinae musculature. The laminae and left transverse process of the lumbar vertebrae were removed with bone cutters in a caudal to rostral direction. Thin layers of gauze soaked in warm Ringer's solution were used to keep the dura moist and small strips of foam impregnated with anticoagulant (Gelfoam ^{R}) were used to prevent bleeding from cut surfaces of the bone.

The animal was suspended in a spinal frame ('Canberra') by 2 clamps on the twelfth thoracic vertebra and ileum with sufficient rostrocaudal tension to allow stable electrophysiological recording. Mediolateral stability was ensured by placement of side clamps apposed to the lateral aspect of the vertebral body about midway between the suspending clamps about one segment rostral to the recording site. A silver chloride coated silver wire was wrapped in gauze and soaked in Ringer's solution and sutured between the muscle and fascia beside a lateral clamp to serve as the reference electrode. In some experiments, the tibial and/or the sural nerve were dissected free and set up under warm mineral oil for stimulation *en passage* with bipolar platinum electrodes. Body temperature, heart rate, blood pressure and end-tidal pCO_2 were continuously monitored. The body temperature was maintained between 36 - 38°C with a heating pad controlled by a thermistor placed between a scapula and rib cage. If blood pressure fell below 100 mm of Hg in the absence of drugs, a 'plasma expander' such as dextran was administered. Cats were artificially ventilated following muscular paralysis with gallamine triethiodide (Flaxedil^{R}, 4.0 mg/kg initially) and the tidal volume was adjusted to give an end-tidal pCO_2 of 4.0 ± 0.2 %. In initial experiments the dura mater was slit rostro-caudally to expose

the pia mater , but later experiments were performed with the dura intact except for small perforations in the meninges for microprobe insertion. The intact dural preparation seemed to prevent tissue swelling throughout the course of the experiment perhaps by a retention of the oncotic tissue pressure. In both instances a pool of warm, sterile Ringer's solution was maintained above the cord.

Cold Block Experiments

In the majority of experiments the spinal cord was transected at the thoracolumbar junction following infusion of 2% lignocaine solution (Xylocaine^{R}) to prevent acute stimulatory effects, while the experiments with the cold-block apparatus produced a reversible block of spinal conduction. The concave surface of the metal chamber was placed in close apposition to the dura mater and the edges sealed with silicone grease. A mixture of ethylene glycol - water was circulated through the chamber at either 38°C for normal bulbospinal conduction or - 2°C for blockade of conduction in dorsal columns. This apparatus produced a stable temperature of 2 - 4°C at the dorsal cord surface under the metal chamber sufficient to block responses of lumbar dorsal horn neurones to cervical cord electrical stimulation (Duggan *et al.* 1977a). Sufficient interruption of bulbospinal conduction was confirmed by a decrease in blood pressure upon circulation of cooling fluid through the apparatus.

Morphine Dependence Experiments

In some experiments, cats were treated twice daily for three days with morphine according to a method previously shown to produce tolerance and dependence (Johnson & Duggan, 1981). The dose schedule was 2, 5, 5, 10, 10, 20 mg/kg i.p. morphine HCl with a final dose of 20 mg/kg given before induction of anaesthesia with pentobarbitone (20 mg/kg, i.p.).

Silanation of Glass Micropipettes

In initial experiments, hollow precision-bore glass blanks (Pyrex^R, borosilicate) of 3mm outer diameter were used in the silanation procedure. After overnight treatment in 50% nitric acid, rinsing in distilled water and drying in an oven, the blanks were drawn out under heat and tension in a microelectrode puller to give shanks that were flexible enough to withstand subsequent treatments. The large end was sealed in the flame of a Bunsen burner and the tip was sealed by heating with a small electric coil under a microscope. In later experiments, solid glass blanks (Pyrex^R, borosilicate) were preferred for their finer taper and more flexible nature with the added advantage of eliminating 2 sealing steps. The preferred geometry was 30 μm at 1.5 mm from the tip and 50 μm at 3.0 mm from the tip.

The new glass surface of the shanks was acid-leached by immersing micropipettes in 50% nitric acid for 15 min, which promotes the formation of silanol groups on the glass surface (see **Fig. 1**). Residual acid was removed by three washes in distilled water and the micropipettes were dried in an oven at 140°C.

Various methods have been attempted to increase the proportion of glass micropipettes receiving an even granular coating of siloxane polymer. One method involves a hydration step to direct the polymerization of silane to the surface of the silanol groups, which may involve water molecules in the reactive intermediate (Kopaciewicz & Regnier, 1986).

To achieve a surface water film the micropipettes were cooled to - 20°C and transferred to a 10% solution of 3-aminopropyltriethoxysilane in toluene through a humid environment. They remained for 24 h in the silane solution until removed to a wash of toluene, and heated in an oven at 140°C for 24 h. This heating cures the polymer to a stable, water insoluble form and maximizes the number of amine groups free from steric hindrance (Chiang *et al.* 1982). At this stage individual probes were inspected under a microscope for imperfection

Fig. 1. The silanation of glass micropipettes.

A. Low magnification light microscopic images before (left) and after (right) silane treatment of glass micropipettes.

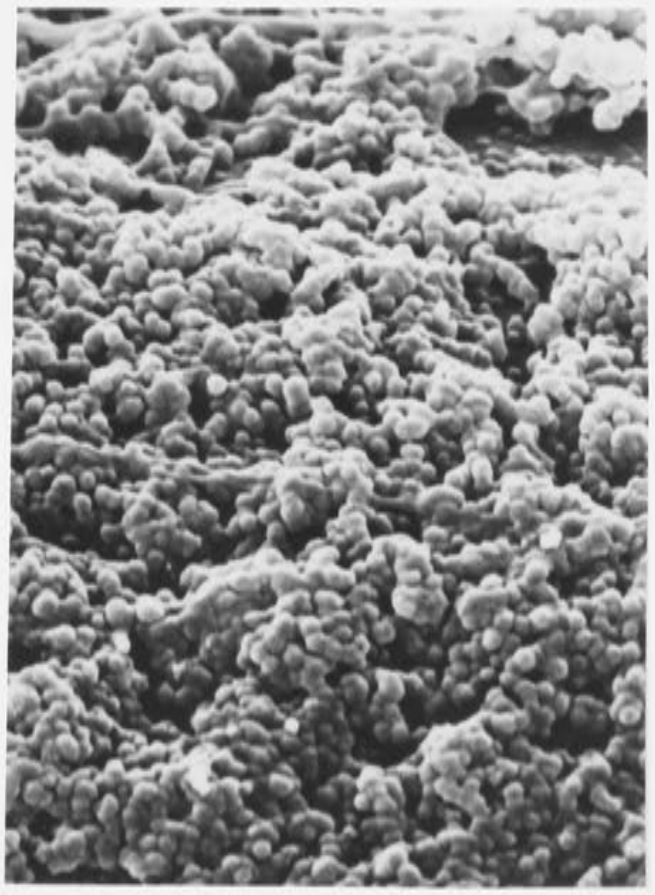
B. High magnification scanning electromicrograph of the outer surface of silane-treated micropipette.

Lower part of this figure shows the chemical structures involved in the silanation reaction.

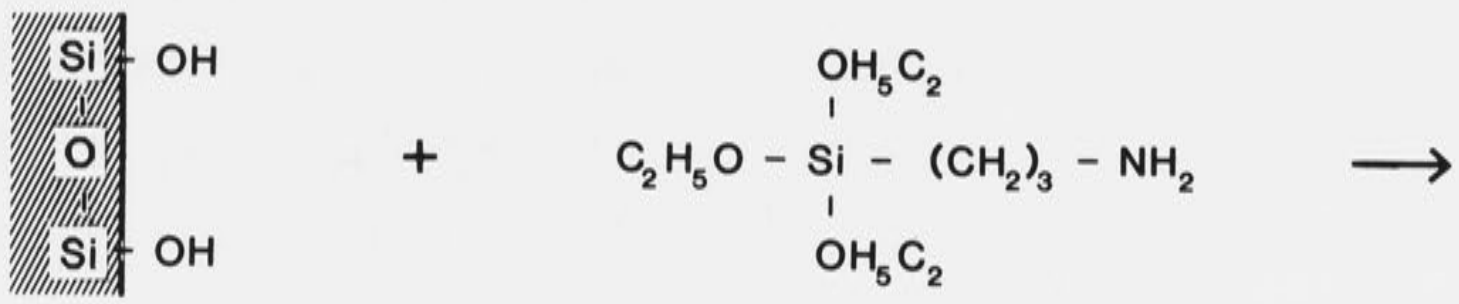
A

100μm

B

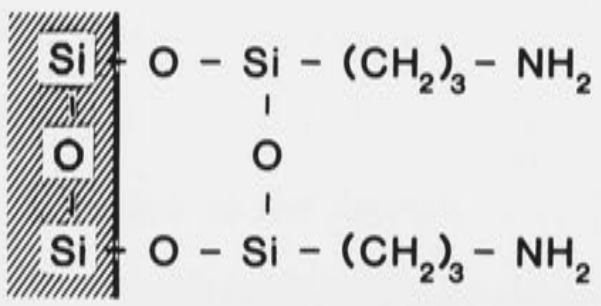


1μm



GLASS

γ-AMINOPROPYLTRIETHOXYSILANE



ALKYLAMINE GLASS

and graded according to thickness and quality of polymer (**Fig. 1**). The silanation procedure was repeated up to 5 times until the desired number of good quality probes were obtained.

Preparation of Antibody Microprobes

The technique discussed in this section is summarized in **Fig. 2.** A cross-linking molecule was reacted with the free amine groups of the siloxane polymer by immersing micropipettes in a 2.5%(v/v) aqueous solution of glutaraldehyde (Merck) for 30 min followed by three washes of distilled water.

Protein A was bound onto the surface of the polymer to adsorb selectively the IgG fraction of the antisera (Goding, 1978). A concentration of 2 mg/ml Protein A (Sigma) was taken up into 5 μl microcapillary tubes and individual microprobes were inserted into the solution with the aid of a dissecting microscope, then incubated for 24 - 48 h at 6°C in a glass dessicator with 100 % humidity. Micropipettes were then immersed in a 2.5%(w/v) solution of sodium borohydride to reduce Schiff bases formed during glutaraldehyde coupling of the Protein A to the polymer (Royer *et al.* 1975). Following three rinses in PBS the micropipettes were inserted into 5 μl capillaries containing an appropriate dilution of antiserum (see **Table I**) with the aid of a dissecting microscope, and put in a humid environment at 6°C for 24 - 48 h.

In Vitro Assays

In theory, the optimal dilution of antiserum to use for a sensitive radioimmunoassay with a low detection limit is the first in a series of normalised antibody dilution standard curves which no longer shifts to the left in proportion to the dilution of antiserum (Hunter, 1983). In practice, this means enough antibody sites available to give a good signal-to-noise ratio, but not so many that a high concentration of the neuropeptide under investigation

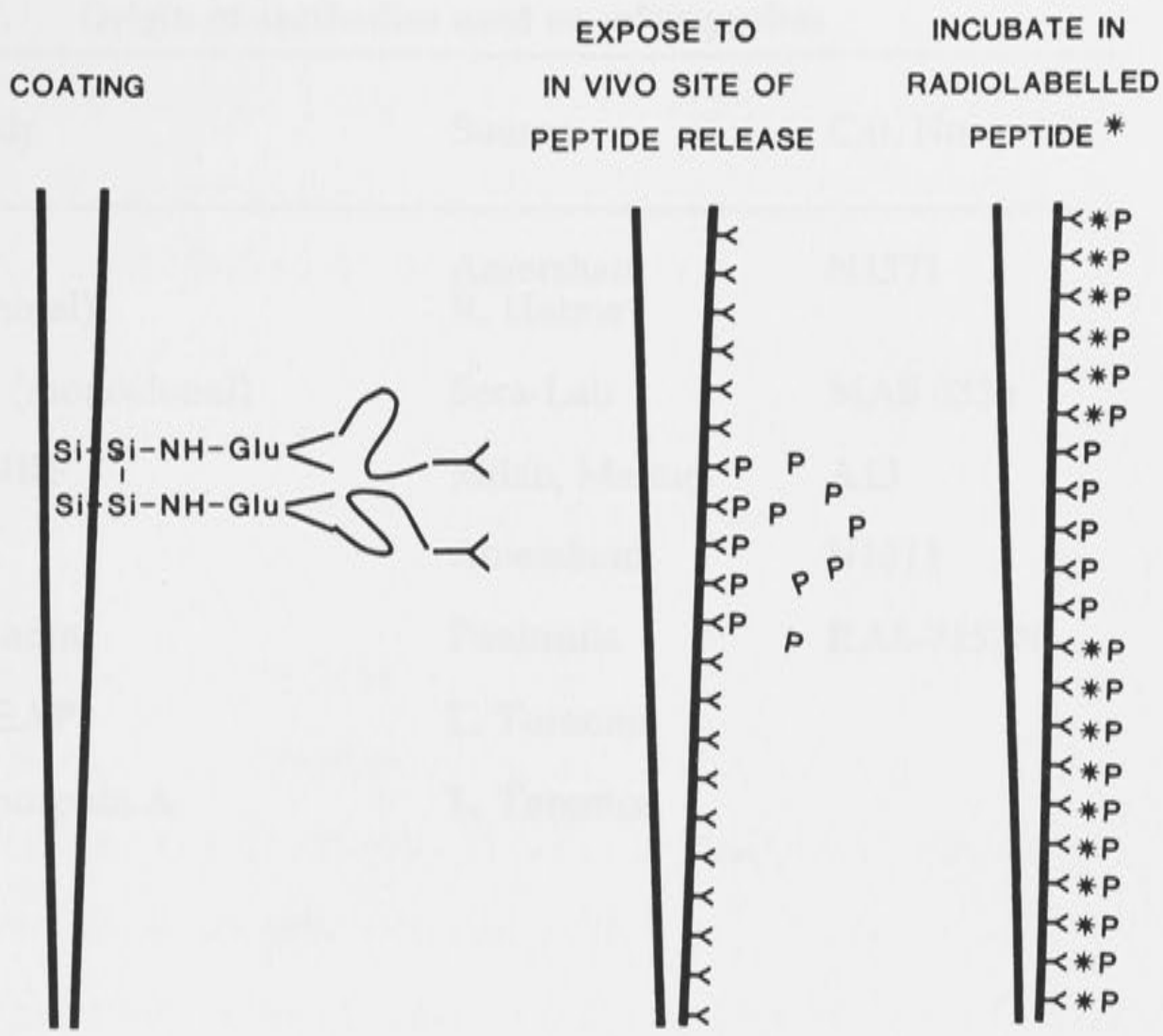


Fig. 2. Diagram of the preparation and use of antibody microprobes to detect release of sensory neuropeptides.
Glu, glutaraldehyde; **P**, peptide antigen; ***P**, radiolabelled peptide.
Below: Simplified diagram of the image analysis system shown in **Fig. 3.**.

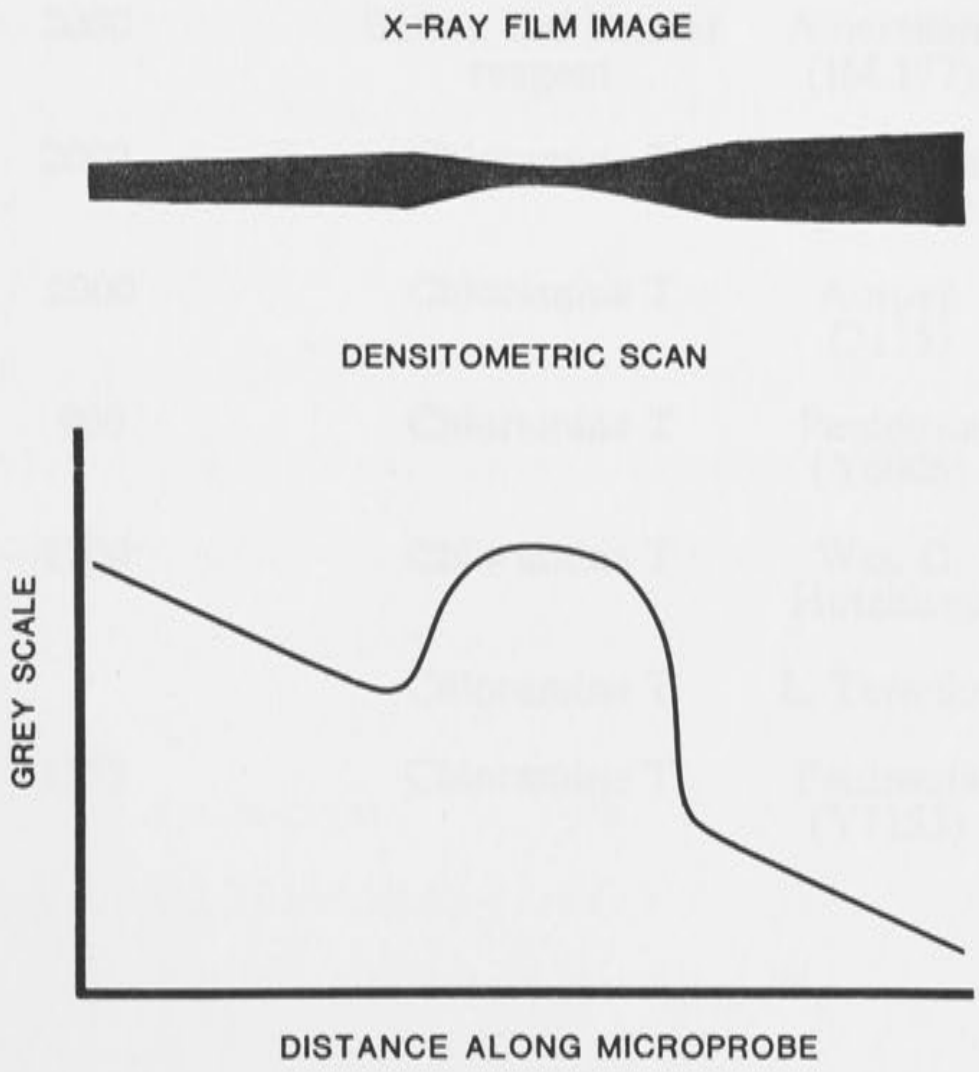


Table I. Origin of antibodies used on microprobes

Antibody	Source	Cat. No.
anti-SP (C-terminal)	Amersham R. Helme	N1571
anti-SP (monoclonal)	Sera-Lab	MAS 035b
anti-CGRP	Milab, Malmö	A13
anti-SS	Amersham	N1611
anti-galanin	Peninsula	RAS-7153N
anti-MEAP	L. Terenius	
anti-dynorphin A	L. Terenius	

Table II. Radiotracers used in antibody microprobe experiments.

Tracer	Specific Activity (Ci/mmol)	Iodination Method	Source & Cat. No.
^{125}-I-SP	2000	Bolton and Hunter reagent	Amersham (IM.157)
^{125}I-tyr^{11}-SS	2000	Chloramine T	Amersham (IM.161)
^{125}I-CGRP(rat)	2000	Chloramine T	Auspep (2115)
^{125}I-tyr^{0}-CGRP	900	Chloramine T	Peninsula (Y6008)
^{125}I-dyn A_{1-17}	1700	Chloramine T	Wm. D. Hutchison
^{125}I-MEAP	?	Chloramine T	L. Terenius
^{125}I-Galanin	1375	Chloramine T	Peninsula (Y7153)

is required to displace the radiolabelled form of the neuropeptide. To find the optimal antiserum concentration, *in vitro* assays were carried out by preparing microprobes with increasing dilutions of antiserum with and without known concentrations of synthetic neuropeptide to obtain standard curves. Radioiodinated peptides were obtained commercially (see **Table II**) except for dynorphin A which was prepared by the chloramine T procedure (Hunter, 1974) and purified on a Sep-Pak C-18 cartridge (Waters).

Experimental Use of Antibody Microprobes

On the day of experimentation , individual probes were again microscopically inspected to ensure adequacy of coating, numbered for identification and immersed in cold PBS azide.

To determine the appropriate somatotopic areas of the cord for microprobe placement, a platinum ball electrode was used to record the maximum cord dorsum potentials in response to low threshold stimulation of an ipsilateral tibial or sural nerve, or to light mechanical stimulation of the hind paw. Prior to microprobe insertion, a 4 M NaCl-filled microelectrode was used to record excitatory responses of neurones in laminae IV/V of the same spinal segment to light mechanical cutaneous stimuli and low intensity nerve stimulation.

Micromanipulators (Narishige, Japan and Transvertex stepping drive, Sweden) were used to insert microprobes into the lower lumbar spinal cord to particular depths beneath the dorsal cord surface. Microprobes were inserted about midway between the lateral and median dorsal sulci with slight variations in mediolaterality depending on the pattern of the venous plexus. At the end of some experiments, a micropipette filled with pontamine sky blue (a 2% solution in 1.2 M sodium acetate) was inserted to the depth of insertion of the antibody microprobes and current was passed to eject dye as an anion from the elctrode tip, to confirm the depth of insertion by subsequent histology.

Thermal, mechanical or chemical cutaneous stimulation of the ipsilateral hind paw was given for specific periods ranging from 5 min to 60 min, and usually 10 - 20 min. The hind paw was immersed in a waterbath at 35 - 42°C for non-noxious, or 50 - 52°C for noxious thermal stimulation. Small bulldog clips were used to pinch a fold of skin of the ipsilateral digital pads, and this was repeated (*i.e.* 2 min 'on', 1 min 'off') throughout the time period of microprobe insertion. Methylene chloride in a polymer base (commercial paint stripper) was applied to the skin for chemical stimulation of nociceptors. The tibial or sural nerves were stimulated in some experiments usually with trains of 3 pulses of 0.5 ms width at 333 Hz, repeated at 10 Hz for 10 - 20 min, and occasionally at 100 Hz continuous, at an intensity sufficient to excite A$\alpha\beta$ (1.5 x T), Aδ (11 - 16 x T) or additionally, C afferent fibres (200 - 300 x T). In a few cases, hollow antibody microprobes were used to record excitatory responses in lamina V/VI to cutaneous stimuli applied to the ipsilateral hind paw.

Upon removal from the cord, antibody microprobes were washed in PBS azide containing 0.2% Tween for 15 min and inserted into 5μl capillary tubes containing about 2,000 cpm/μl of the radiolabelled peptide (**Table II**) for 24-48 h at 6°C.

A final washing step in PBS-Tween for 20 min was carried out to remove non-specific radiolabel binding. The terminal 15 - 20 mm of each antibody microprobe was removed from the glass shaft and was fixed to graph paper with white typing correction fluid (Liquid Paper^{R}), lining up in sequence *in vivo* and *in vitro* probes. The paper was placed in an autoradiographic cassette (DuPont) next to X-ray film (Kodak NMC - monoemulsion) with a radiation intensifying screen. The exposure time was estimated from the total binding on *in vitro* probes and was in the order of 2 - 5 days, and further exposures were taken to obtain the desired silver grain density on the autoradiographic images. The optical density of silver grains over time of radioactive exposure was found to be linear over the range of exposure times used to obtain autoradiographic

images. The appearance of zones of inhibition of radiotracer binding were interpreted as sites of release of the neuropeptide in question.

Analysis of Autoradiographic Images

A highly sensitive and precise method of image analysis was used to quantify the autoradiographic images to allow averaging of individual image scans within groups and comparison of average image scans between control and experimental groups (see **Fig. 3**). This technology has been described (Hendry *et al.* 1988) and employs a video camera linked to an IBM-AT computer using a modified version of the Autoradiographic Video Image Digitizing System (AVID, see (Hendry *et al.* 1988)).

The film with autoradiographic images was placed on a remote-controlled stage (model number MCC12/20JS-RS232, Lang Electronik, Hüttenberg) lit from beneath by a diffuse incandescent light source controlled by a stabilized DC power supply (model 275, BWD Instruments, Melb.). A inverted, fixed video camera (Videcon, Dage D565) fitted with a micro lens (Nikon Bellows PB-6) was focussed from above on an unexposed portion of film and the light was adjusted to 250 on an arbitrary grey scale of 0 (dark) to 255 (light). With this apparatus, this corresponded to a power supply to the light source of 35 V and 650 mA. A background reading was digitized in duplicate and stored in computer memory for subsequent subtraction from image scans. The same magnification was used for each probe inserted to the same depth in the cord. For example, autoradiographic images of probes inserted 3.00 mm in the spinal cord all had 3.8 mm scanned and digitized. Upper and lower limits enclosing 40 pixels were centred around the image and greyscale values were totaled for each of 240 divisions of 16 μm width along the length of the image (see **Fig. 3**). In experiments with CGRP, narrow greyscale additions across only 4 pixels down the centre of the image were used in an attempt to increase the

Fig. 3. Sequential stages of computer analysis of autoradiographic images.

A. Video screen image of microprobe autoradiograph.

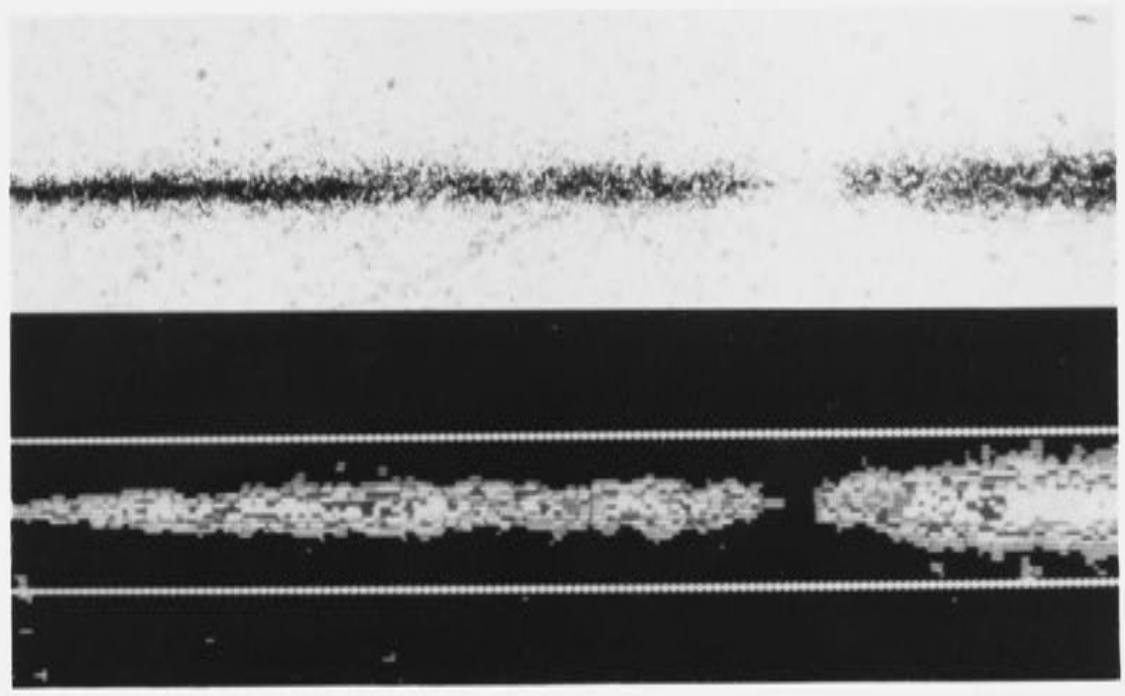
B. Black and white photograph of the digitized colour image showing upper and lower limits for image integration. The image is not as sharply defined as in A. because the analysis system has detected minor changes in optical density and assigned grey scale values to them, appearing as false colours on the screen.

C. The image density scan of a single antibody microprobe detecting irSP release.

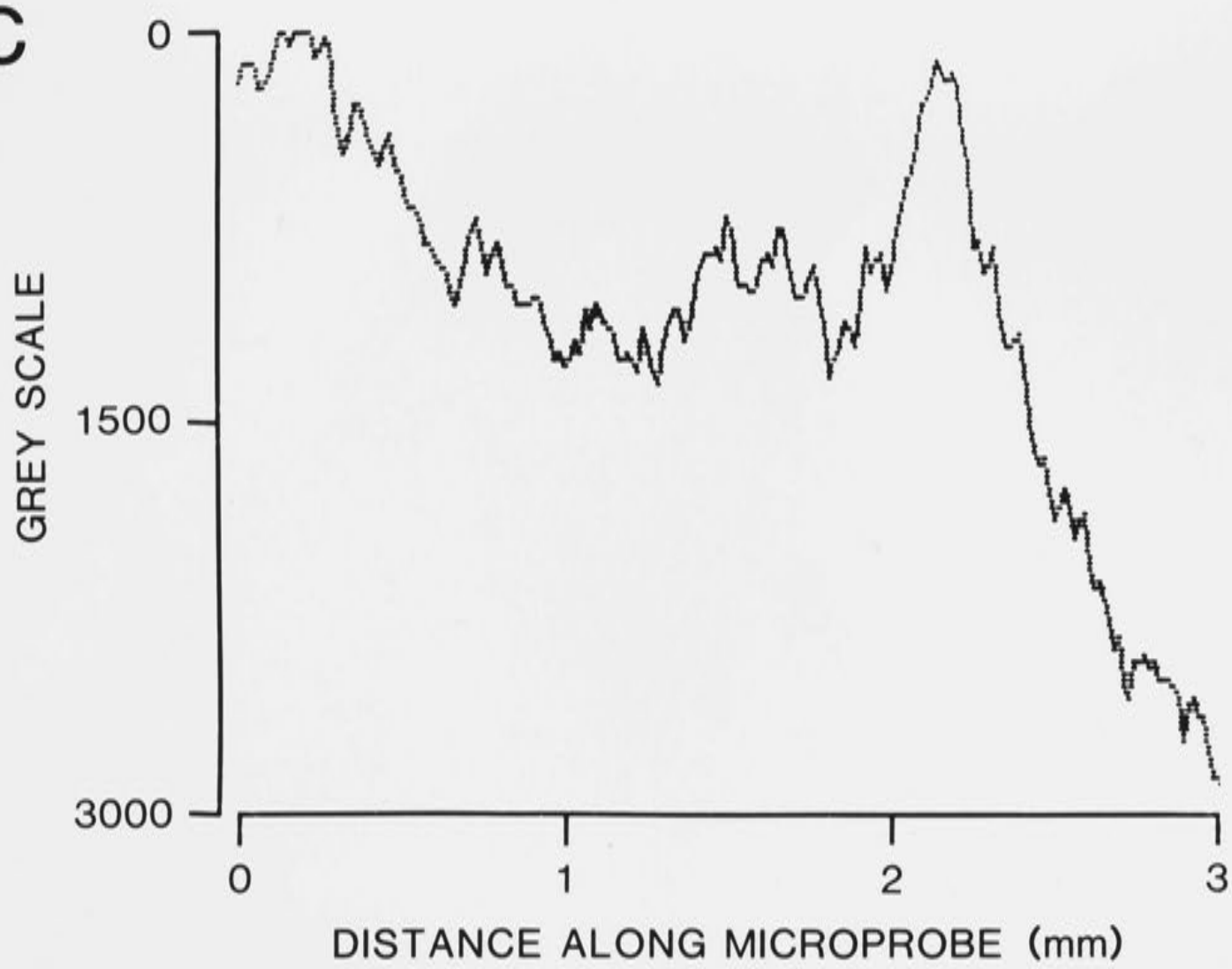
D. (Following Page) A colour enlargement of a digitized image of a microprobe scan like that shown in B.

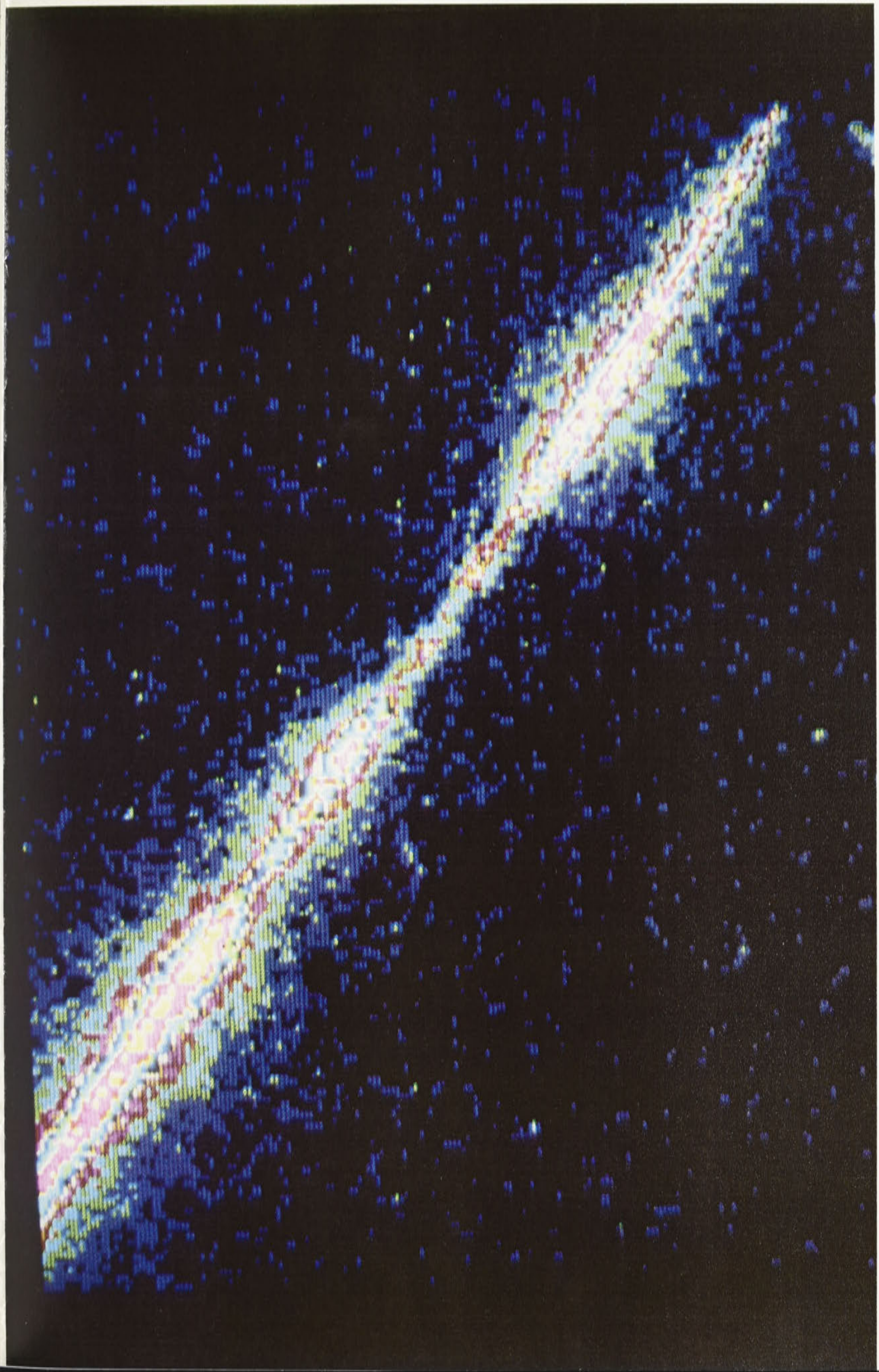
A

B



C





signal to noise ratio but little difference was seen in peak height over the background when compared to wide greyscale additions of 40 pixels.

Each peptide investigated was stored in a separate data base file, with individual scans identified with a code for general information of date, experiment number, exposure time and more specific information such as time in cord, type of physiological stimuli and drug doses. Groups were collected by computer searches of code numbers and a mean greyscale value was plotted as a solid line with the SEM as broken lines.

An initial comparison between the two groups was provided by the subtraction of the mean scan line for the control group from the experimental group. Any deviation from the zero line indicated a difference in the zone of release which could be tested for significance by a linear plot of the t-statistic for each point. Further analysis was performed by determining the area under the scan (AUS) corresponding to the zone of release for individual scans within a group (see **Fig. 4**), and comparing the mean AUS between groups. Other programmes were used to edit sections of individual scans that were due to imperfections of the silane coating or of tracer binding.

Immunohistochemistry

In some experiments, a verification of the presence of the peptide was sought using immunohistochemistry with the same antibodies that were immobilized on the microprobes. At the end of antibody microprobe experiments, three deeply anaesthetized cats were given an intravenous injection of 5 ml of 3% (v/v) heparin in saline, a saturated KCl solution, and perfused intracardially with 500 ml of cold PBS followed by 500 ml of 4% paraformaldehyde. The lumbar spinal cord was removed and immersed in 4% paraformaldehyde for several days. Before cutting sections the spinal cord was immersed overnight in 10% sucrose in PBS azide as a cryoprotectant. Colchicine was not used in these experiments to enhance cell body staining

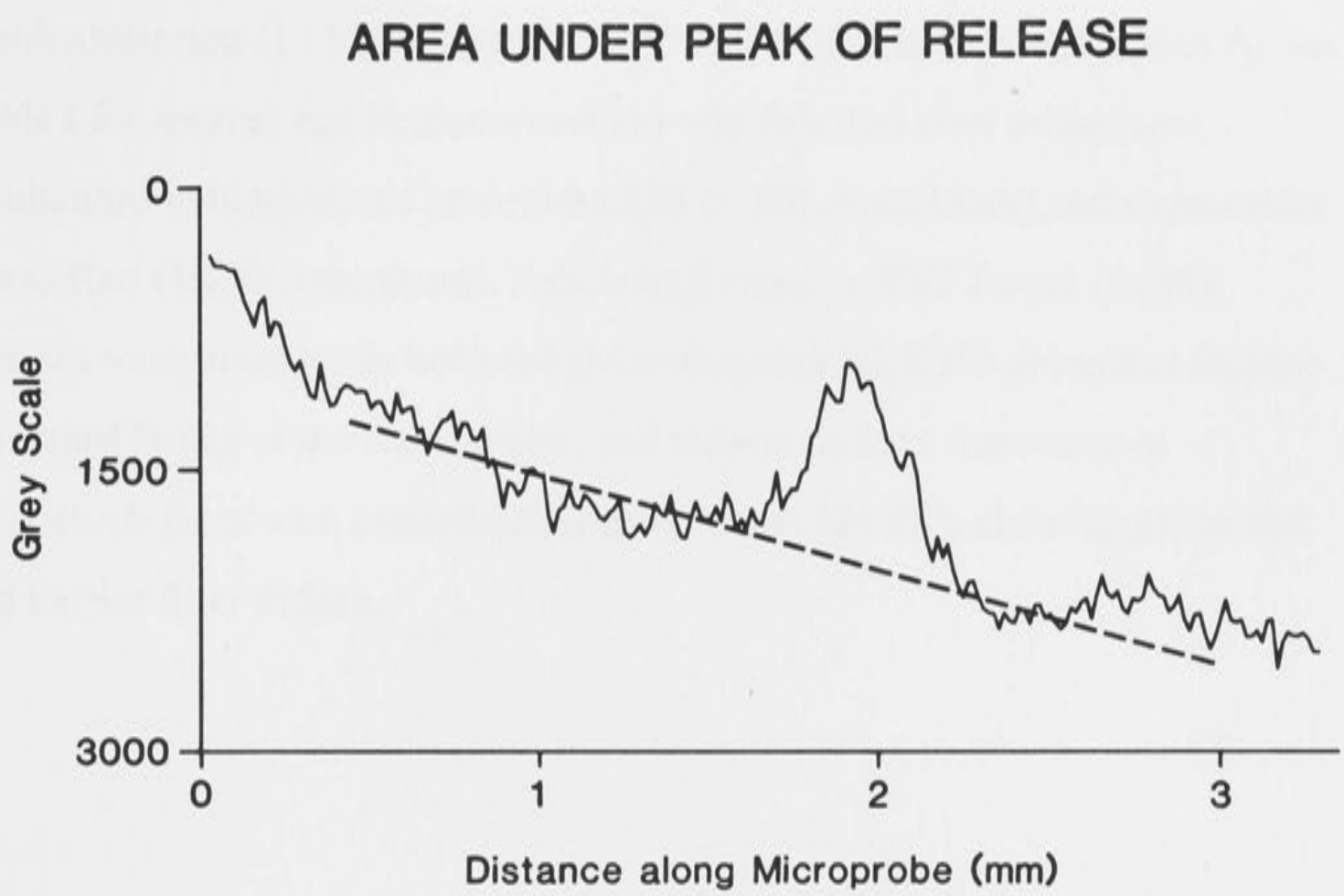


Fig. 4. Method for calculating the area under the scan (AUS).
An imaginary line is drawn along the base of the scan and two points are selected on the line which best describe the base of the area to be calculated. A computer programme shades in the area and calculates the value in arbitrary greyscale x mm square units.

since this treatment may have decreased peptide immunoreactivity at the sites of release in nerve terminals. Transverse 20-50μm sections were cut on a freezing microtome and were incubated in PBS Tween (0.2%) containing 10% non-immune horse serum. Sections were incubated overnight at 4°C in the rabbit antiserum (1 : 1,250 for anti-CGRP and 1 : 600 for anti-dynorphin A; see **Table I** for source) and immunoreactivity was detected after subsequent incubations in biotinylated anti-rabbit IgG (1:100, Amersham) and streptavidin Texas-Red (1:100, Amersham). Following 3 rinses in PBS Tween (0.1%), sections were mounted in buffered glycerol containing 0.1% phenylenediamine (to retard fading of the fluorophore) and viewed under a fluorescence microscope fitted with excitation-bandpass filters 530-585, dichroic mirror 600 and barrier filter 615nm.

4. STIMULI EVOKING RELEASE OF NEUROPEPTIDES IN THE SPINAL CORD

Release of Substance P

Results

A total of 463 microprobes form the basis of this analysis. Of these, 447 were inserted into the spinal cords of 34 cats to vertical depths of 2.0 to 3.0 mm from the dorsal surface for 10, 15, 20 or 30 min.

Basal release

When performing experiments on animals subjected to extensive lumbar surgery and nerve dissections of the hind limb, it was important to examine antibody microprobes inserted into the lumbar spinal cord without any deliberate peripheral stimulus to assess the level of 'basal' release.

Fig. 5A shows the mean image density scan of 16 microprobes not exposed to SP *in vitro* prior to incubation in 125I-SP. Total binding of the labelled peptide produces a near uniform slope due to the taper of individual probes. Fig. 5B illustrates the mean image density scan for 31 microprobes inserted 3mm into the spinal cord for periods of 10 to 30 min under conditions of no peripheral stimulation. The difference between these in vivo probes and the zero SP in vitro probes is shown in Fig. 5C. The subtraction produced an upward deflection representing a zone of release of irSP on the microprobes. A similar result was observed with 37 microprobes inserted 2.0 mm and other depths into the spinal cord (not illustrated).

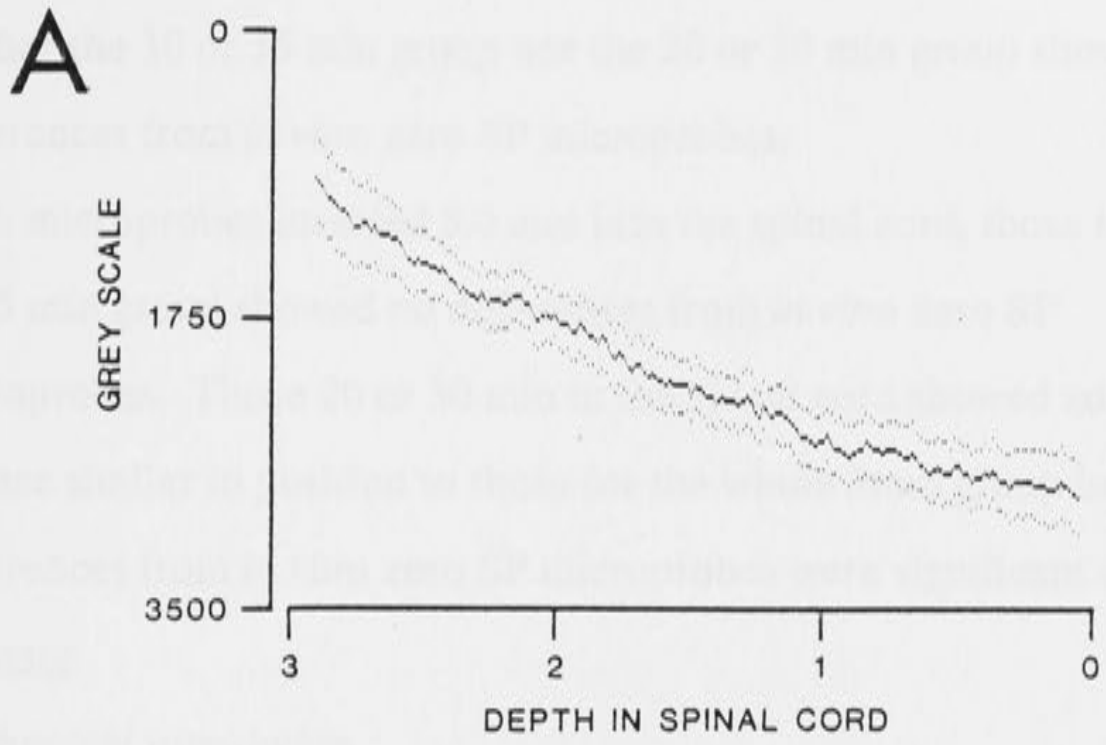
Two peaks are visible: one just below the surface of the spinal cord and one centered on a point 1.2 mm from the cord surface corresponding to the region of the substantia gelatinosa. These differences were not significant ($P > 0.05$) when considering data from all experiments (**Fig. 5C**).

These differences were further analysed in terms of length of time in the spinal cord. This can be summarized as follows:

Fig. 5. Spontaneous release of irSP in the anaesthetized cat.
A. *In vitro* controls. The mean image density scan of 16 microprobes incubated in ^{125}I-SP without prior exposure to unlabelled SP is plotted with respect to length. The terminal 3 mm has been analysed in 16 μm intervals with the tip positioned at 0 and both the mean and standard errors of the mean at each point are illustrated. Optical density is expressed as an arbitrary grey scale (see Methods).

B. The mean image density scan of 31 microprobes inserted 3 mm into the spinal cord without added peripheral stimulation.
Ordinate: optical density as in A.
Abscissa: depth in the spinal cord (mm), tips being at 3.
Analysis of the terminal 200 μm has been deleted.

C. The differences between mean scans A and B and the calculated t values for the differences of the means. The broken line is the t value indicating significance at $P = 0.05$. The difference has been performed such that release on the *in vivo* probes is represented as an upward deflection.


A
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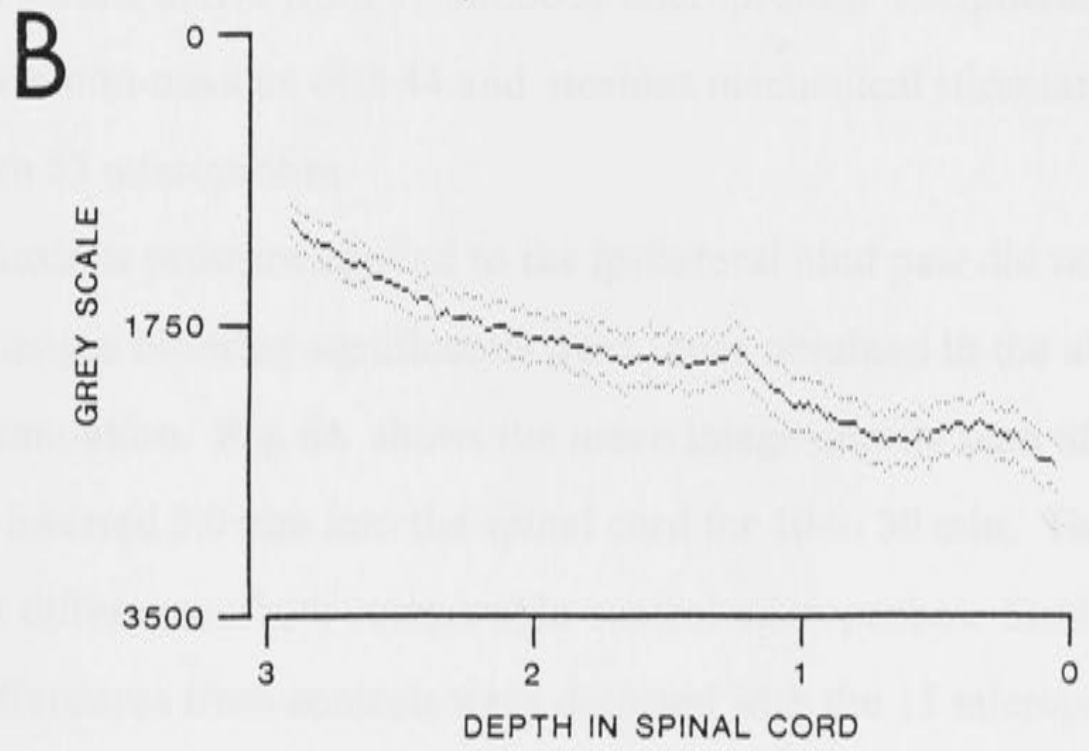
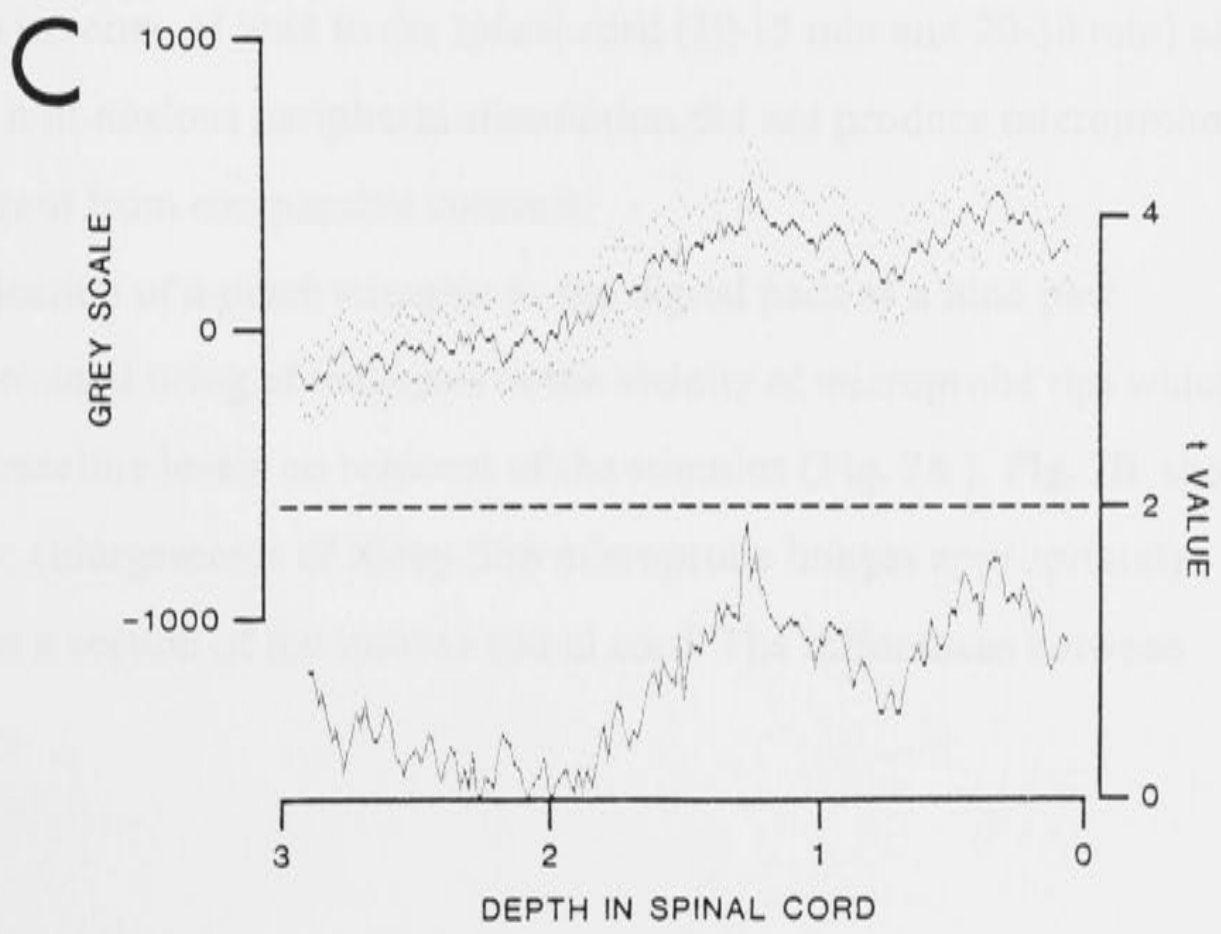
1750
3500
GREY SCALE
3
2
1
0
DEPTH IN SPINAL CORD


B
0
1750
3500
GREY SCALE
3
2
1
0
DEPTH IN SPINAL CORD


C
1000
0
-1000
GREY SCALE
4
2
0
t VALUE
3
2
1
0
DEPTH IN SPINAL CORD

(a) With microprobes inserted 2.0 mm and other depths into the spinal cord neither the 10 or 15 min group nor the 20 or 30 min group showed differences from *in vitro* zero SP microprobes.

(b) With microprobes inserted 3.0 mm into the spinal cord, those in the 10 or 15 min group showed no differences from *in vitro* zero SP microprobes. Those 20 or 30 min in the spinal cord showed zones of release similar in position to those for the whole 3mm group but these differences from *in vitro* zero SP microprobes were significant ($P < 0.05$).

Evoked release

Mechanical stimulation

These results derive from 97 antibody microprobes. Peripheral stimulation was non-noxious with 44 and noxious mechanical stimulation was employed with 53 microprobes.

Non-noxious pressure applied to the ipsilateral hind paw did not produce microprobe images differing significantly from those obtained in the absence of peripheral stimulation. **Fig. 6A** shows the mean image density scan of 29 microprobes inserted 3.0 mm into the spinal cord for 10 to 30 min. There are no significant differences from comparable control microprobes. Similarly, no significant differences from controls were detected with the 15 microprobes inserted 2.0 mm into the spinal cord (not illustrated). Further analysis of microprobes in terms of time in the spinal cord (10-15 min and 20-30 min) also showed that non-noxious peripheral stimulation did not produce microprobe images different from comparable controls.

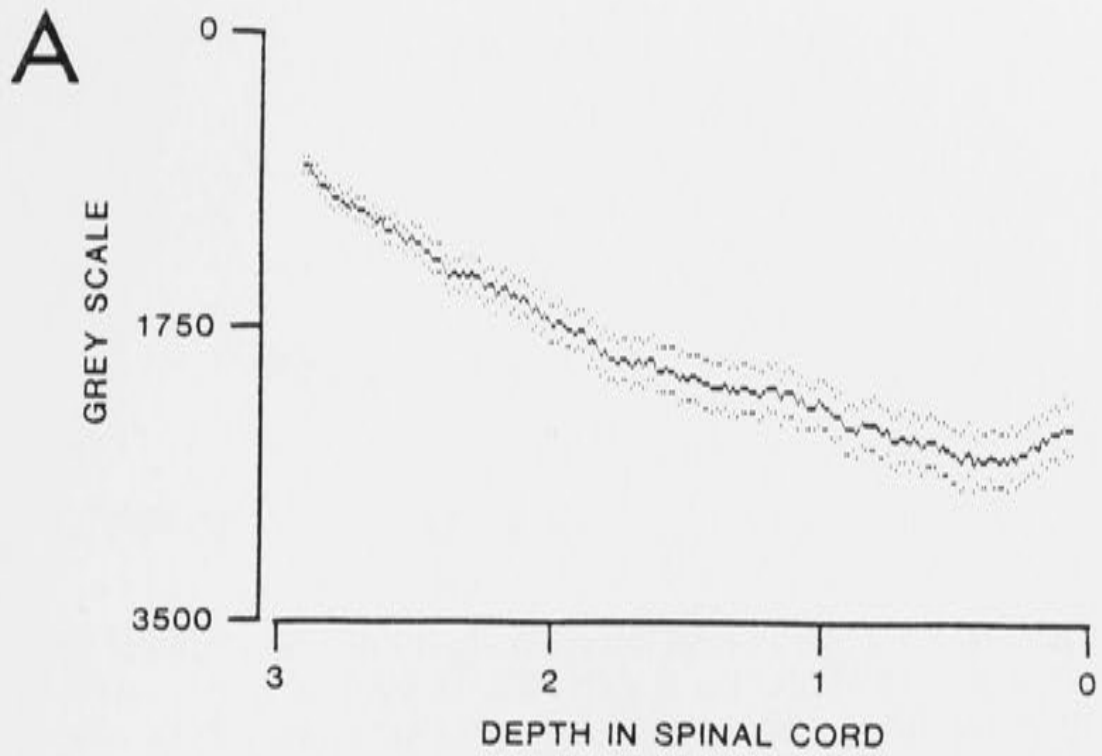
Application of a pinch stimulus to the digital pads of a hind paw produced sustained firing of neurones in the vicinity of microprobe tips which returned to baseline levels on removal of the stimulus (**Fig. 7A**). **Fig. 7B** shows photographic enlargements of X-ray film microprobe images appropriately positioned on a section of the lumbar spinal cord. The differences between

Fig. 6. Release of irSP by noxious mechanical peripheral stimuli. Ordinates and abscissae as for **Fig. 5.**

A. The mean image density scan of 29 microprobes inserted 3 mm into the spinal cord during non-noxious mechanical peripheral stimulation for 10 to 30 min.

B. The mean image density scan of 22 microprobes inserted 3 mm into the spinal cord for 10 to 30 min during noxious mechanical peripheral stimulation.

C. The differences between the mean scans plotted in A and B and the t value calculated for these differences. The lower line is the t value indicating significance at the 0.05 level.


A
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1750
3500
GREY SCALE
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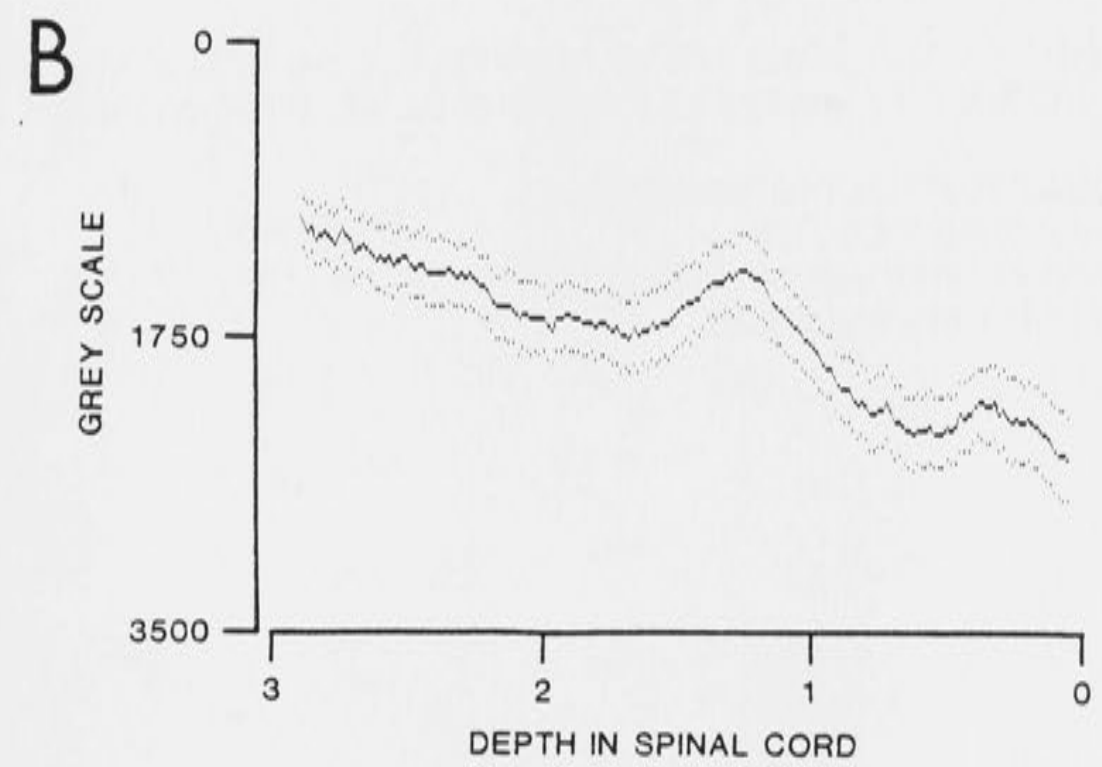
0
DEPTH IN SPINAL CORD


B
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1750
3500
GREY SCALE
3
2
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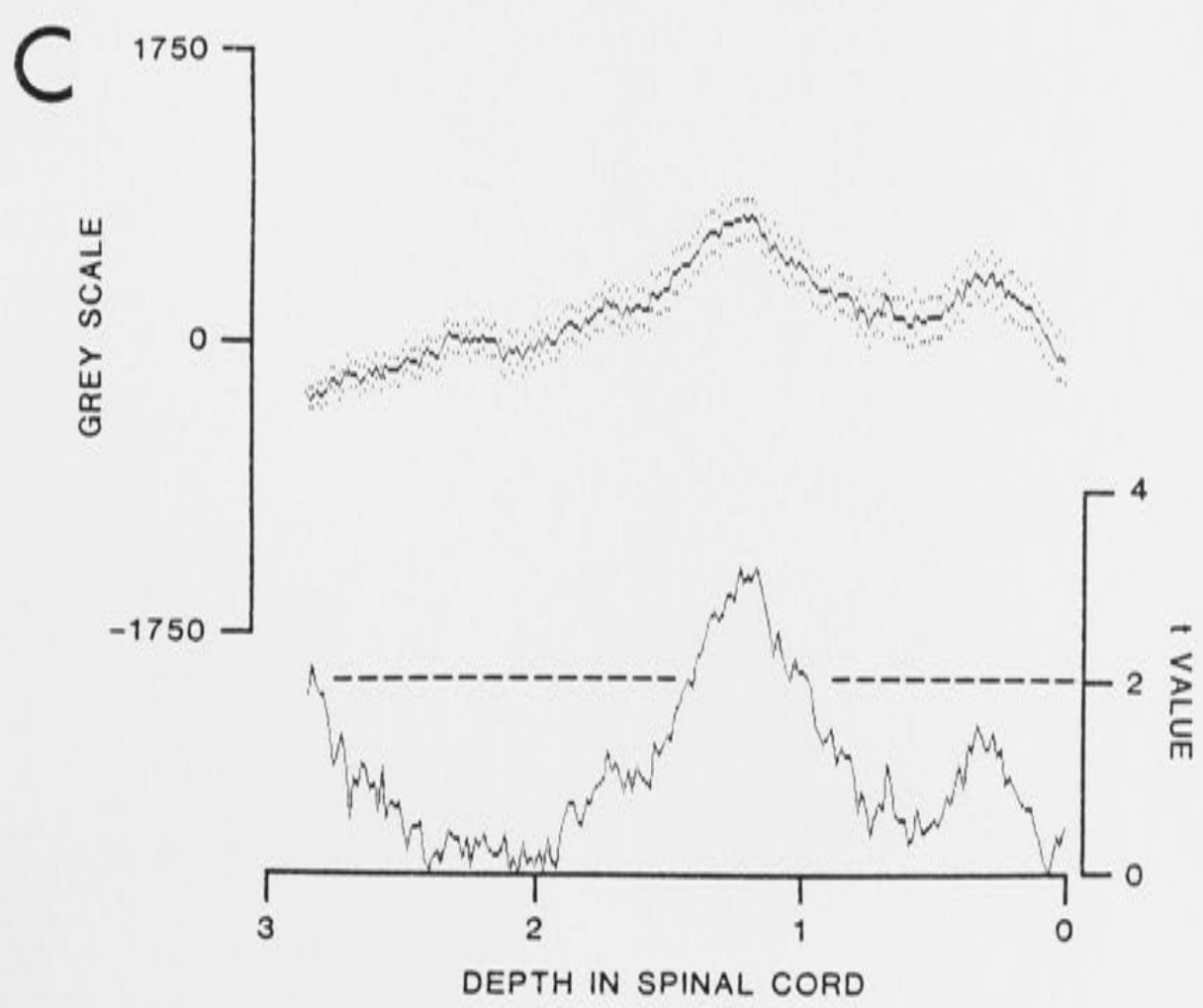
0
DEPTH IN SPINAL CORD


C
1750
0
-1750
GREY SCALE
4
2
0
t VALUE
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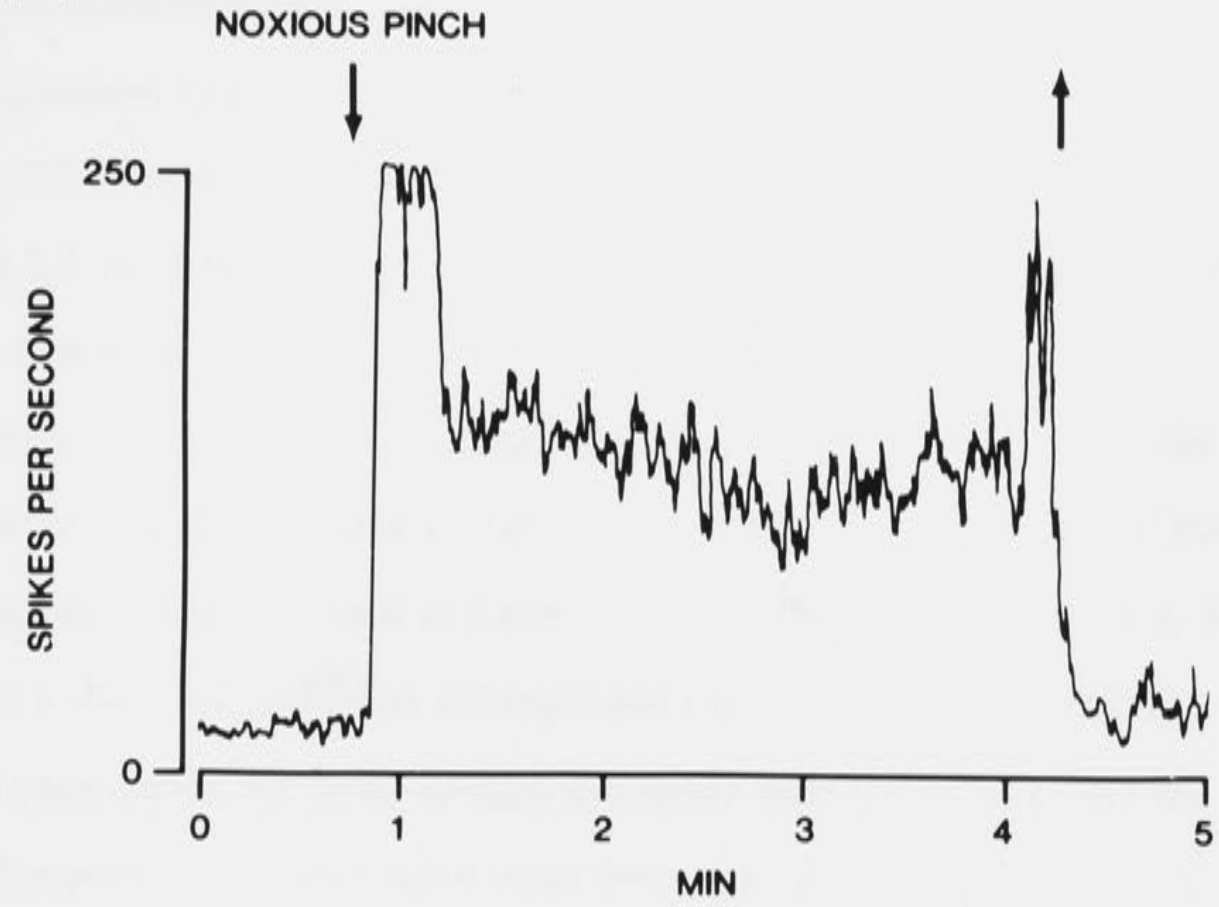
2
1
0
DEPTH IN SPINAL CORD

Fig. 7A. Ratemeter record of multiunit firing to noxious mechanical stimulation recorded with a microprobe inserted 2 mm into the spinal cord. Note the increases in firing associated with application and removal of the alligator clips as indicated by arrows. Sustained firing was recorded during the period of noxious stimulation.

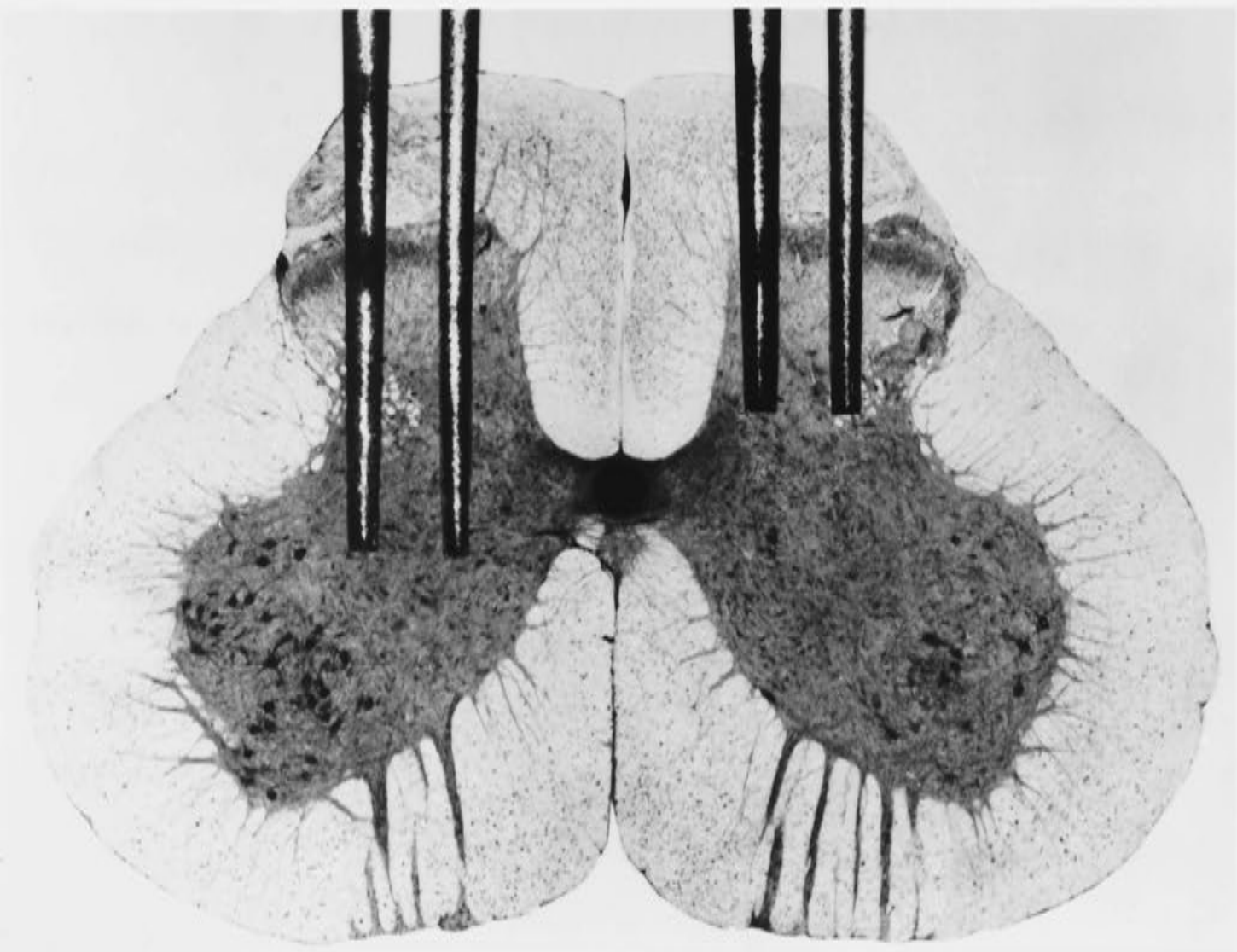
B. Release of irSP by noxious but not by non-noxious peripheral stimuli. Microprobes **a** and **b** were inserted to a depth of 3 mm, microprobes **c** and **d** to 2 mm.

a. 30 min, noxious mechanical stimulation.
b. 20 min, no peripheral stimulus.
c. 20 min, noxious mechanical stimulation.
d. 30 min, no peripheral stimulation.

A



B



microprobes inserted under conditions of noxious and non-noxious mechanical stimulation are readily visible.

Fig. 6B shows two clear zones of release of irSP, one just beneath the spinal cord surface and one larger peak centered 1.2 mm from the cord surface. **Fig. 6C** is the difference between the mean scans for noxious and that for non-noxious mechanical stimulation, and thus represents the effect of the noxious component of the mechanical stimulation. Analysis of 31 microprobes inserted 2.0 mm and 2.5 mm into the spinal cord also showed significant release of irSP at the same regions.

Microprobe images were also analysed in terms of time in the spinal cord. Significant zones of release at the cord surface and in the region of the substantia gelatinosa were found at times in the cord of 10 or 15 min (**Fig. 8A**). Because there were relatively few microprobes representing non-noxious mechanical stimulation for 10 or 15 min, the mean scan for appropriate no-stimulus microprobes has been subtracted from that of noxious mechanical stimulation microprobes. Of interest is a third area of irSP release peaking at a site 2.2 mm from the cord surface, in the region of lamina VI. This can also be seen in **Fig. 6B** but in both cases the zone did not achieve significance.

Heat stimulation

Comparisons have been made of probes inserted into the dorsal horn under conditions of no stimulation, with the ipsilateral hind paw in water at 36°C to 44°C and with water temperatures of 46, 48, 50 and 52°C.

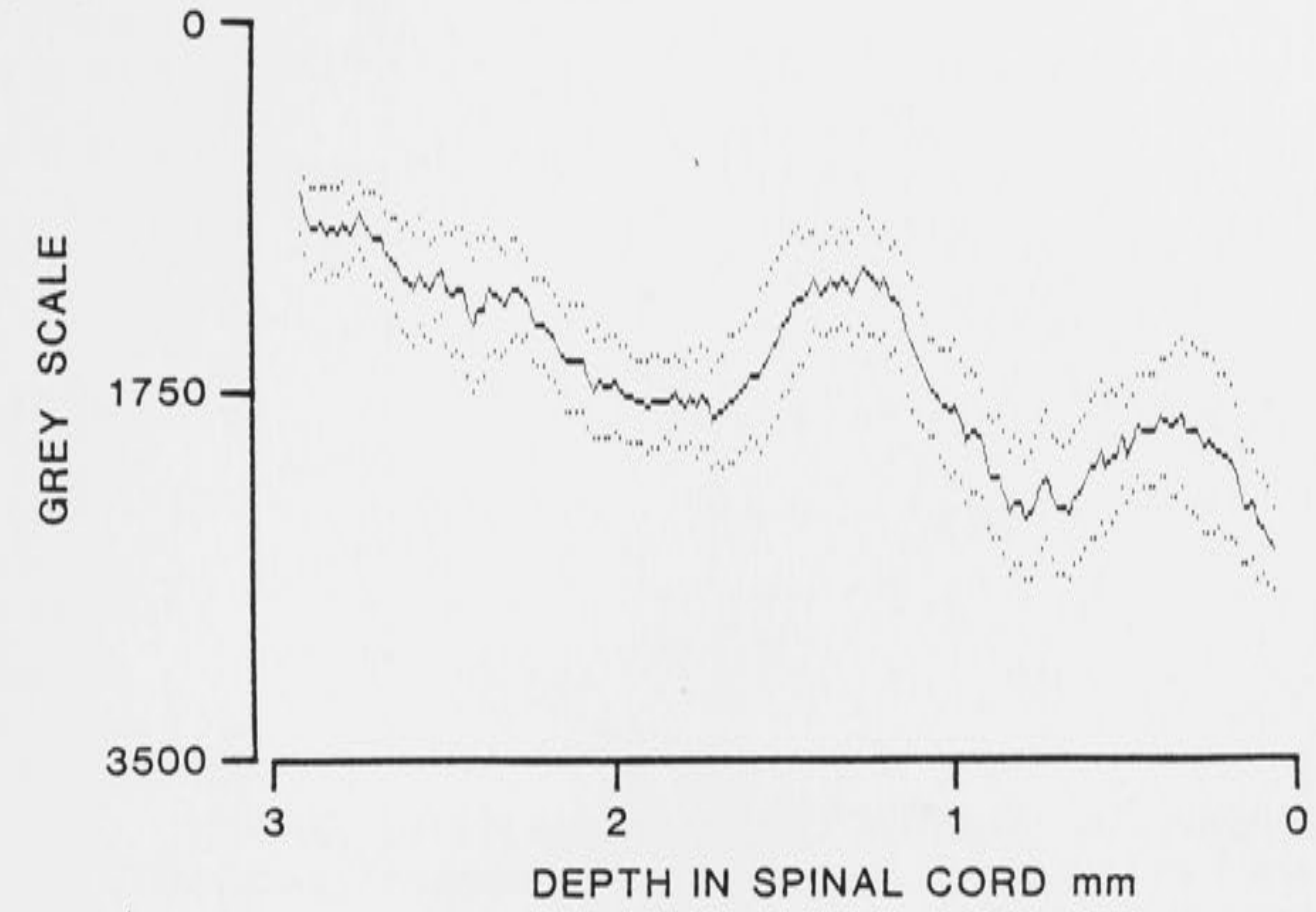
A total of 159 antibody microprobes have been analyzed. The effect of increasing the water temperature on the release of irSP to 47 microprobes inserted 3mm into the dorsal horn for 10 to 30 min is shown in **Fig. 9.** With water in the range 36 to 44°C (non-noxious) the mean image scan was not significantly different from that of control microprobes (limb in air at 23°C). This was also observed with microprobes inserted 2.0 mm into the dorsal horn

Fig. 8. Release of irSP by 10 or 15 min noxious mechanical peripheral stimuli.

A. The mean image density scan of 6 microprobes inserted 3 mm into the spinal cord during 10 or 15 min of noxious mechanical stimulation.

B. Differences between the mean image density scan of 6 microprobes inserted 3 mm into the spinal cord for 10 or 15 min during noxious mechanical stimulation and the mean scan for microprobes inserted to the same depth and for similar times but in the absence of stimulation (n=11). The t values for the differences between means is also plotted. The lower line is the t value indicating significance (P=0.05).

A


0
1750
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GREY SCALE
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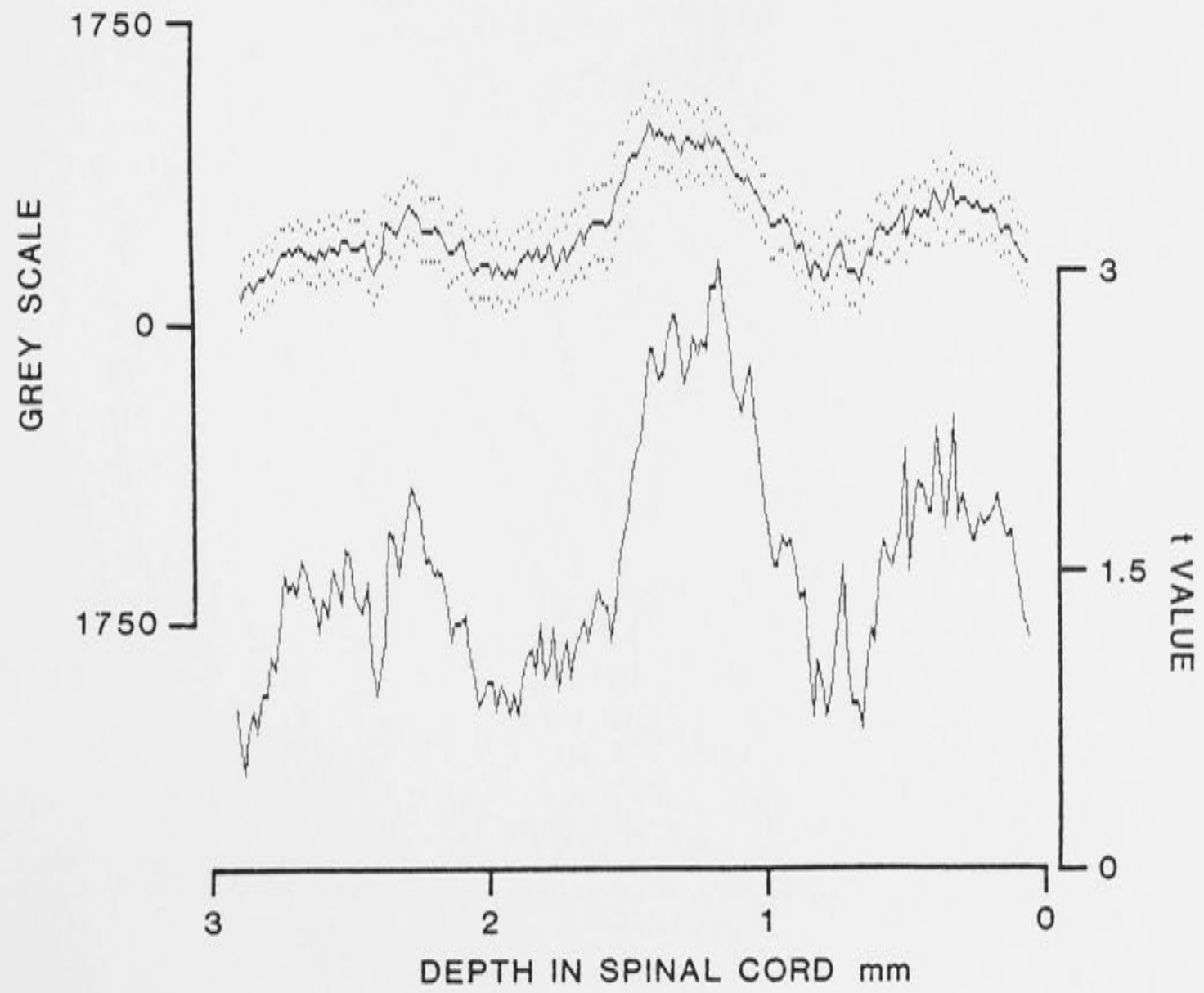
DEPTH IN SPINAL CORD mm

B


1750
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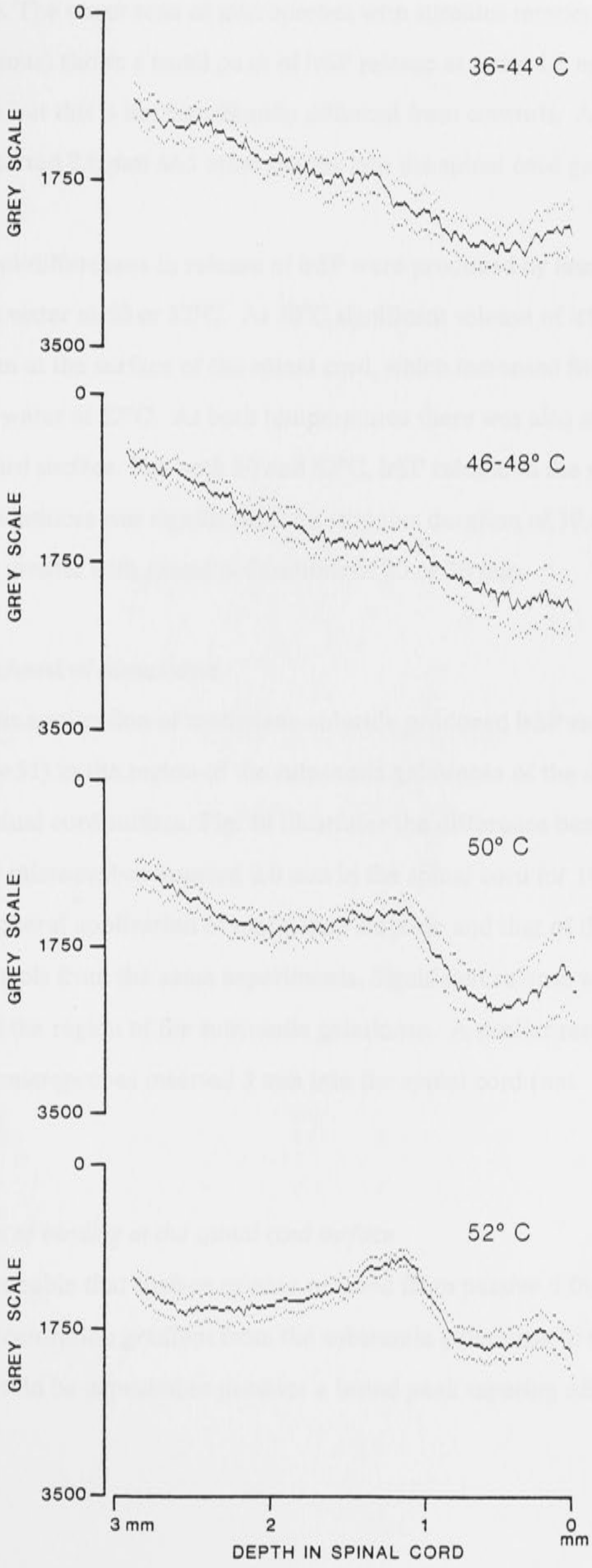
DEPTH IN SPINAL CORD mm

Fig. 9. Increasing thermal stimuli and irSP release in the dorsal horn. Illustrated are the mean image density scans for microprobes inserted 3 mm into the spinal cord for 10 to 30 min during immersion of a hind paw in water at 36-44^{O}C (n=9), 46-48^{O}C (n=9), 50^{O}C (n=9) and 52^{O}C (n=20).


0
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36-44° C
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GREY SCALE
46-48° C
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GREY SCALE
50° C
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1750
3500
GREY SCALE
52° C
3 mm
2
1
0
mm
DEPTH IN SPINAL CORD

(not illustrated). The mean scan of microprobes with stimulus temperatures of 46 to 48°C (noxious) shows a small peak of irSP release at a site 1.2 mm from the cord surface but this is not significantly different from controls. Analysis of microprobes inserted 2.0 mm and other depths into the spinal cord gave similar results.

Significant differences in release of irSP were produced by immersing the hind limb in water at 50 or 52°C. At 50°C significant release of irSP appeared 1.2 mm at the surface of the spinal cord, which increased further with the hind paw in water at 52°C. At both temperatures there was also significant release at the cord surface. For both 50 and 52°C, irSP release in the region of the substantia gelatinosa was significant for a stimulus duration of 10 or 15 min, and release was greater with stimulus durations of 20 or 30 min.

Noxious chemical stimulation

Cutaneous application of methylene chloride produced irSP release onto microprobes (n=51) in the region of the substantia gelatinosa of the dorsal horn, and the spinal cord surface. **Fig. 10** illustrates the difference between the mean scan of 29 microprobes inserted 2.0 mm in the spinal cord for 10 to 30 min during peripheral application of methylene chloride and that of the appropriate controls from the same experiments. Significant release was obtained only in the region of the substantia gelatinosa. A similar result was obtained for 22 microprobes inserted 3 mm into the spinal cord (not illustrated).

Inhibition of binding at the spinal cord surface

It is improbable that surface release resulted from passive diffusion of irSP down a concentration gradient from the substantia gelatinosa to the dorsal surface. This would be expected to produce a broad peak tapering off towards

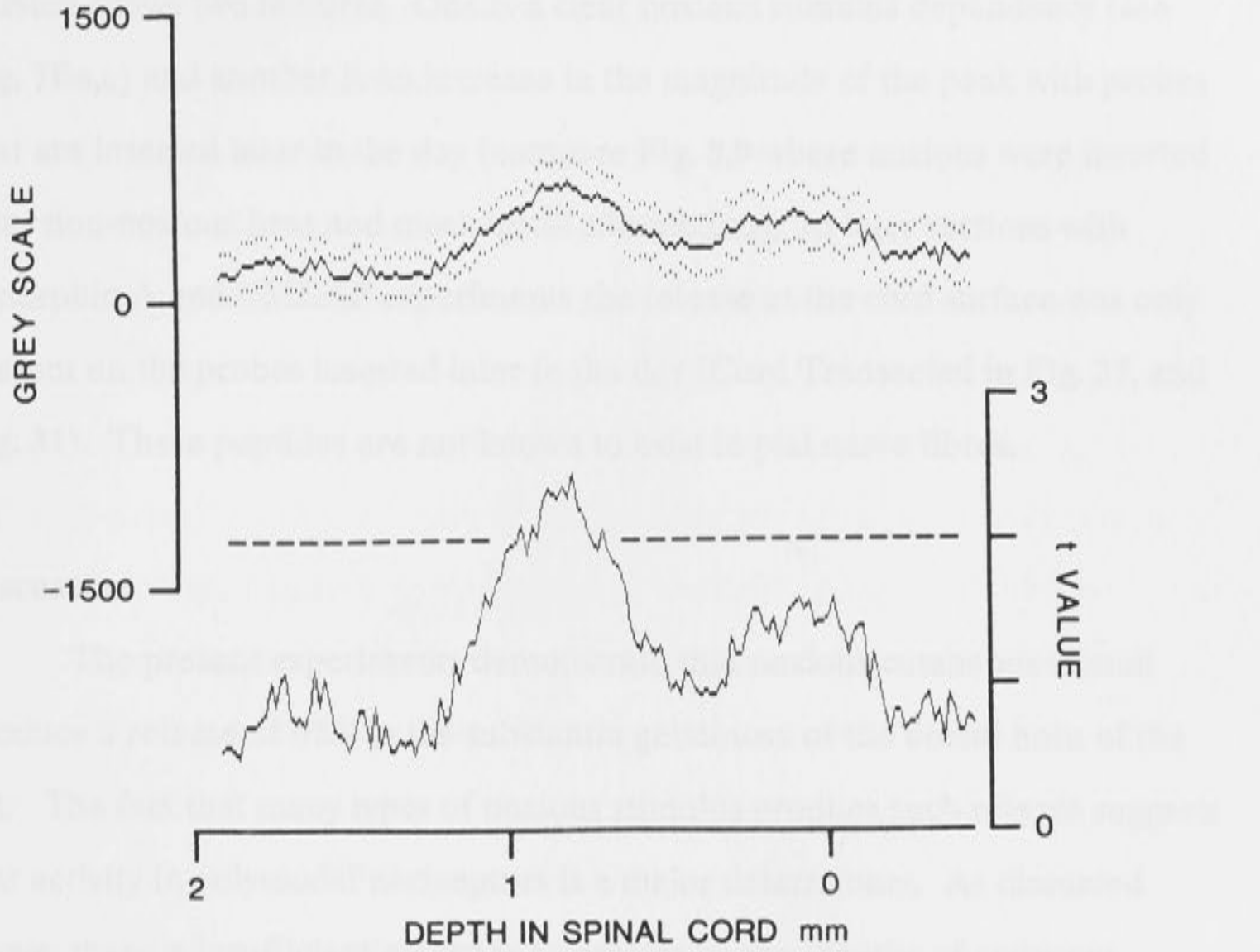


Fig. 10. Release of irSP in the dorsal horn with cutaneous noxious chemical stimulation.
Illustrated is the difference between the mean image density scans for 29 microprobes inserted 2 mm into the spinal cord for 10 to 30 min while **methylene chloride** was applied to the skin of the ipsilateral hind paw and the mean scans for no stimulus, control microprobes from the same experiments. The t values for the differences of the means are also plotted and the lower line is the t value for significance (P=0.05).

the dorsal surface, instead of the two discrete peaks observed in these experiments.

The inhibition of binding of radiotracer at the surface of the cord dorsum shows two features. One is a clear noxious stimulus dependency (see **Fig. 7Ba,c**) and another is an increase in the magnitude of the peak with probes that are inserted later in the day (compare **Fig. 8,9** where noxious were inserted after non-noxious heat and mechanical stimulation). In later sections with dynorphin A and irMEAP experiments the release at the cord surface was only present on the probes inserted later in the day (Cord Transected in **Fig. 27**, and **Fig. 31**). These peptides are not known to exist in pial nerve fibres.

Discussion

The present experiments demonstrate that noxious cutaneous stimuli produce a release of irSP in the substantia gelatinosa of the dorsal horn of the cat. The fact that many types of noxious stimulus produce such release suggests that activity in polymodal nociceptors is a major determinant. As discussed above, there is insufficient evidence to determine the identity of apparent release at the spinal cord surface. The occasional observation of irSP release in deeper laminae suggests that irSP release from specialized nociceptors may also occur, and this is an indication of the spatial precision of the present technique. Each of the sites of release of irSP requires separate discussion.

Release in the region of the substantia gelatinosa

Release in the substantia gelatinosa was not unexpected since this is the major site of termination of unmyelinated nociceptive primary afferents (Light & Perl, 1977; Sugiura *et al.* 1986). The computer-averaged scans of images broaden the area over which apparent release occurs but the peak of this area and the results obtained when single probes were positioned with respect to dye deposits in the spinal cord indicate that it is correct to centre a zone of release

on the substantia gelatinosa. Immunohistochemical studies of the distribution of SP in the dorsal horn have observed relatively high levels in the region of the substantia gelatinosa (Hökfelt *et al.* 1976; Hökfelt *et al.* 1977; Gibson *et al.* 1981; Di Giulio *et al.* 1985), and the binding of radiolabelled SP is also marked in this area (Charlton & Helke, 1985a).

The present experiments cannot identify the structures releasing irSP with certainty but other evidence favours release mainly from the central terminals of primary afferents. Sectioning the dorsal roots lowers the levels of SP in the upper dorsal horn of several species by 50-80% (Takahashi & Otsuka, 1975; Jessell *et al.* 1979; Ogawa *et al.* 1985; Di Giulio *et al.* 1985). Severe localized depletions in the region of lamina I and the substantia gelatinosa as shown by immunohistochemistry (Wall *et al.* 1981; Di Giulio *et al.* 1985) have been produced by sectioning the sciatic nerve, suggesting that the actual loss in deafferented areas is greater than that revealed by analyses of whole dorsal horn. Substance P-containing cell bodies are found in the superficial dorsal horn (Ljungdahl *et al.* 1978; Barber *et al.* 1979) but they are not common. In patients with congenital insensitivity to pain, there is a deficit of small diameter dorsal root ganglion neurones associated with a paucity of SP-containing fibres in the region of the substantia gelatinosa (Pearson *et al.* 1982). Collectively these observations make it highly likely that the greater part of irSP detected after stimuli which produce impulses in unmyelinated primary afferent fibres is released from the central terminals of those fibres.

These results indicate that noxious intensities of thermal, mechanical and chemical stimuli all result in a release of irSP in the region of the substantia gelatinosa. With heating of the ipsilateral hind paw, release was shown to be temperature dependent with no significant release in the range 36 to 48°C but clear release with a temperature of 50°C and more at 52°C. Although preliminary results (Duggan *et al.* 1987) were at variance with (Kuraishi *et al.* 1985), these extended studies have shown agreement in failing to detect

release of irSP with skin temperatures in the range 46 to 48°C. Although there are difficulties in comparing results obtained by heating a localized area of the hind paw and measuring temperatures with an intradermal thermocouple (Kuraishi *et al.* 1985) with those obtained by immersing the whole hind paw in a controlled temperature water bath, it is probable that they failed to detect irSP release following noxious thermal stimuli through the use of insufficiently high skin temperatures.

In accord with the present experiments, (Kuraishi *et al.* 1985) observed a release of irSP in the rabbit dorsal horn with noxious mechanical stimulation. The present experiments have shown that non-noxious mechanical stimulation did not increase irSP release above basal levels and that noxious pinching was required to produce release in the region of the substantia gelatinosa.

The lack of stimulus-specific SP release suggests that polymodal nociceptors contain and release SP. In the rat, irSP has been found in 50% of neurones with conduction velocities in the C fibre range (McCarthy & Lawson, 1989) and in the cat, 30% of a large sample of 131 C fibres were found to be polymodal (Bessou & Perl, 1969). Furthermore, intrathecal SP increases the flexor reflexes of the decerebrate rat to noxious thermal *and* mechanical stimuli (Wiesenfeld-Hallin, 1986b).

Release from stimulus-specific nociceptors may also occur. Leah *et al.* (1985a) examined the relation between function and contained peptide by determining the peptide content of physiologically characterized dorsal root ganglion cells of the cat. Of 15 nociceptors only two contained irSP. Substance P was located in 9 of 12 unidentified unmyelinated primary afferents. These experiments are technically difficult and the results may not be representative of the total population of dorsal root ganglion cells.

Release at the spinal cord surface

The prominence of irSP release at the spinal cord surface was unexpected. The perforation of the dura mater and pia-arachnoid to allow microprobe entry may result in an inflammatory response. In the same year that substance P was first described by von Euler and Gaddum, a description of nerve bundles in the pia mater was reported by Clark (1931). He found axons running in ventral and dorsal pial membranes and found that the innervation was via the ventral roots. More recent work has shown the presence of irSP in pial membranes (Dalsgaard *et al.* 1982) which derives from dorsal and ventral roots. Work by Moskowitz (1985) has shown irSP in cerebral arteries in many species and a release of irSP from bovine pia-arachnoid. In the periphery, SP has also been shown to be released into an inflammatory exudate in the skin (Helme *et al.* 1986; Jonsson *et al.* 1986). Therefore, there may be a substantial contribution of SP from nerves in the pia-arachnoid to surface inhibition of binding of ^{125}I-SP. The presence of other peptides in pial nerves is not as well established as SP. The release of SP from the edges of a pial opening could result in a cascade of events associated with neurogenic inflammation. One of these events which is of concern is the activation of macrophages or mast cells which are plentiful in the meninges, to release prostaglandins and proteolytic enzymes (Hartung *et al.* 1985). There may be some damage to the integrity of antibodies on the microprobe which results in inhibition of radiotracer binding unrelated to the release of the neuropeptide in question. Some evidence suggests that this is occurring. The incidence of patch inhibition is more time dependent than stimulus dependent for most peptides. With SP there does appear to be an early phase of stimulus-dependency of surface inhibition of binding indicating authentic release of SP (Duggan *et al* 1988). It is shown in later sections that surface inhibition was infrequent with SS and CGRP which were inserted for short periods of time (5 - 10 min), and very prominent on irGAL and irMEAP which had longer periods in the cord (30 - 60 min). In

some experiments not presented in this work, antibody microprobes which were incubated in radiotracer *before* insertion in the cord showed similar zones of inhibition of binding, but only at the cord surface. The interpretation of these experiments may be confounded somewhat by the possibilty that enzymes were degrading the radiotracer rather than the antibody, but it does suggest that caution is necessary in interpretation of zones of inhibition of binding at the surface of the cord. In contrast to the erratic nature of appearance of surface 'release', there was still a clear noxious stimulus dependency for release in the region of the substantia gelatinosa.

There was some evidence suggesting a small release of irSP in lamina VI by noxious mechanical but not by noxious thermal and chemical stimuli. Immunocytochemical methods have shown a projection of SP-containing primary afferents to lamina VI (Gibson *et al.* 1981; Jessell, 1982). Our results suggest that these are mechano-nociceptors and not polymodal nociceptors, but further evidence is needed.

Although the present experiments have shown that irSP is released centrally in response to peripheral stimuli, other evidence suggests that some caution is appropriate in ascribing such a neurotransmitter role to the released SP. Among such evidence is the coexistence of SP with other peptides in cat dorsal root ganglion neurones. This is particularly true for calcitonin gene-related peptide but has also been shown for somatostatin, cholecystokinin, vasoactive intestinal polypeptide, and bombesin (Leah *et al.* 1985b; Cameron *et al.* 1988). If substances which coexist in a nerve terminal are co-released then it is possible that the noxious stimuli studied in the present experiments also release a number of peptides in addition to SP. Which one - or which combination of any of these peptides - is responsible for the depolarization of spinal neurones receiving such afferents? Although the antibody microprobe technique does not permit measurement of co-release of peptides contained within one type of neurone, it can be used to detect simultaneous release of

more than one peptide in a particular region of the central nervous system in response to a given peripheral stimulus. In addition, such studies may reveal whether neuropeptides are differentially released in response to defined peripheral stimuli.

Release of Novel Sensory Neuropeptides: CGRP and Galanin

Results

A total of 540 antibody microprobes were analysed in this study. Of these, 290 microprobes were used to detect peptide release *in vivo* (171 for irCGRP, 108 for irGAL and 11 for irSP). The remaining 250 microprobes were used for parallel *in vitro* experiments.

Release of irCGRP

Basal Release

A basal release of irCGRP was observed within the spinal grey matter, in the region of the substantia gelatinosa. **Fig. 11A** illustrated the mean image density scan of microprobes placed in the cord for 10 min in the absence of peripheral stimulation. There is a zone of inhibition of ^{125}I-CGRP binding centered 1.1 mm from the dorsal cord surface, representing basal irCGRP release under these experimental 'no stimulus' conditions. As this level of basal release would enable alterations evoked by peripheral stimulation to be readily observed, most microprobes detecting irCGRP in these experiments were inserted for 10 min periods.

Evoked Release

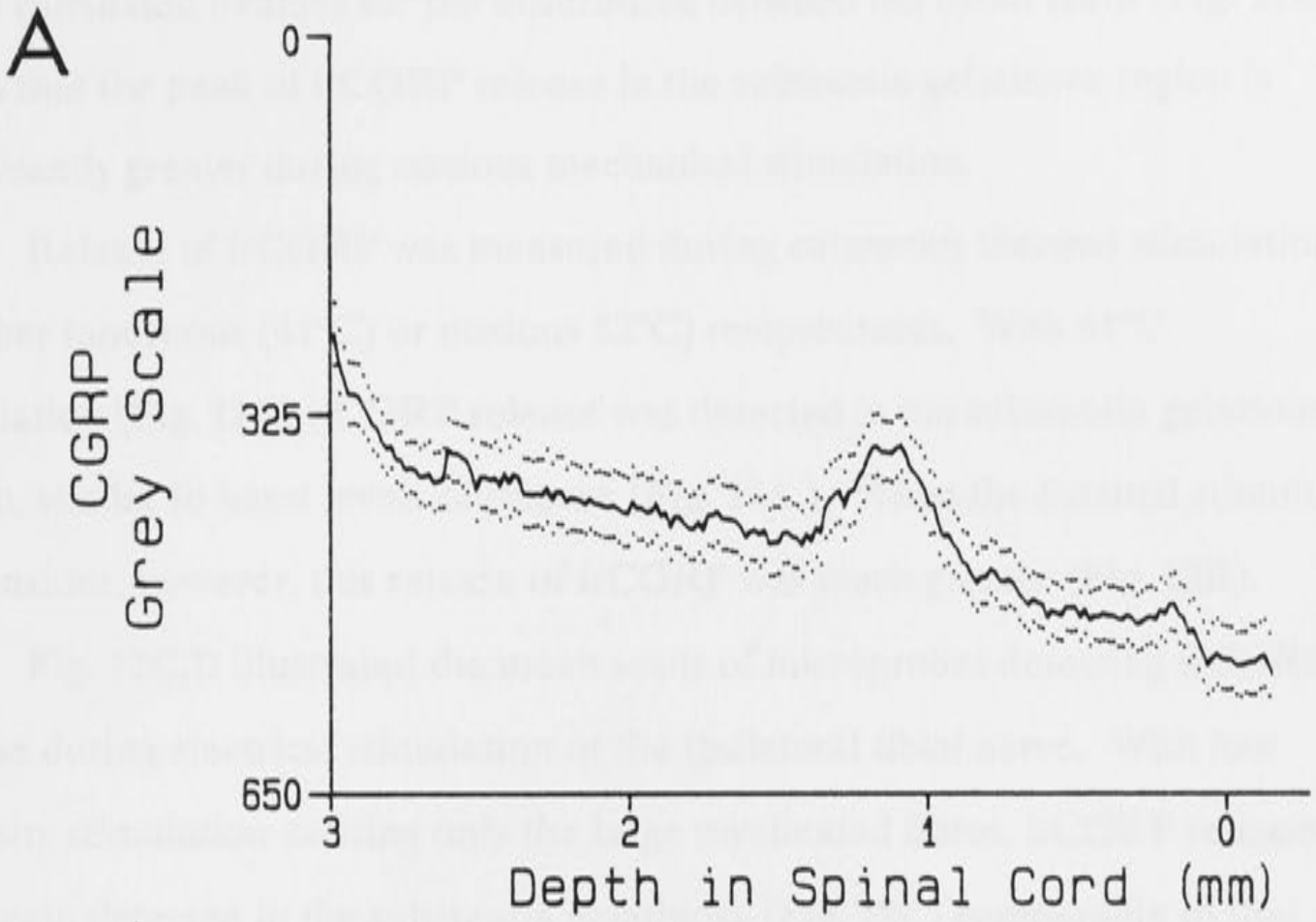
The release of irCGRP in the substantia gelatinosa region was increased by noxious cutaneous mechanical stimulation . **Fig. 11B** shows the mean scan of microprobes placed in the cord during 10 min of such stimulation, plotted together with the mean scan of microprobes detecting basal release. The plot

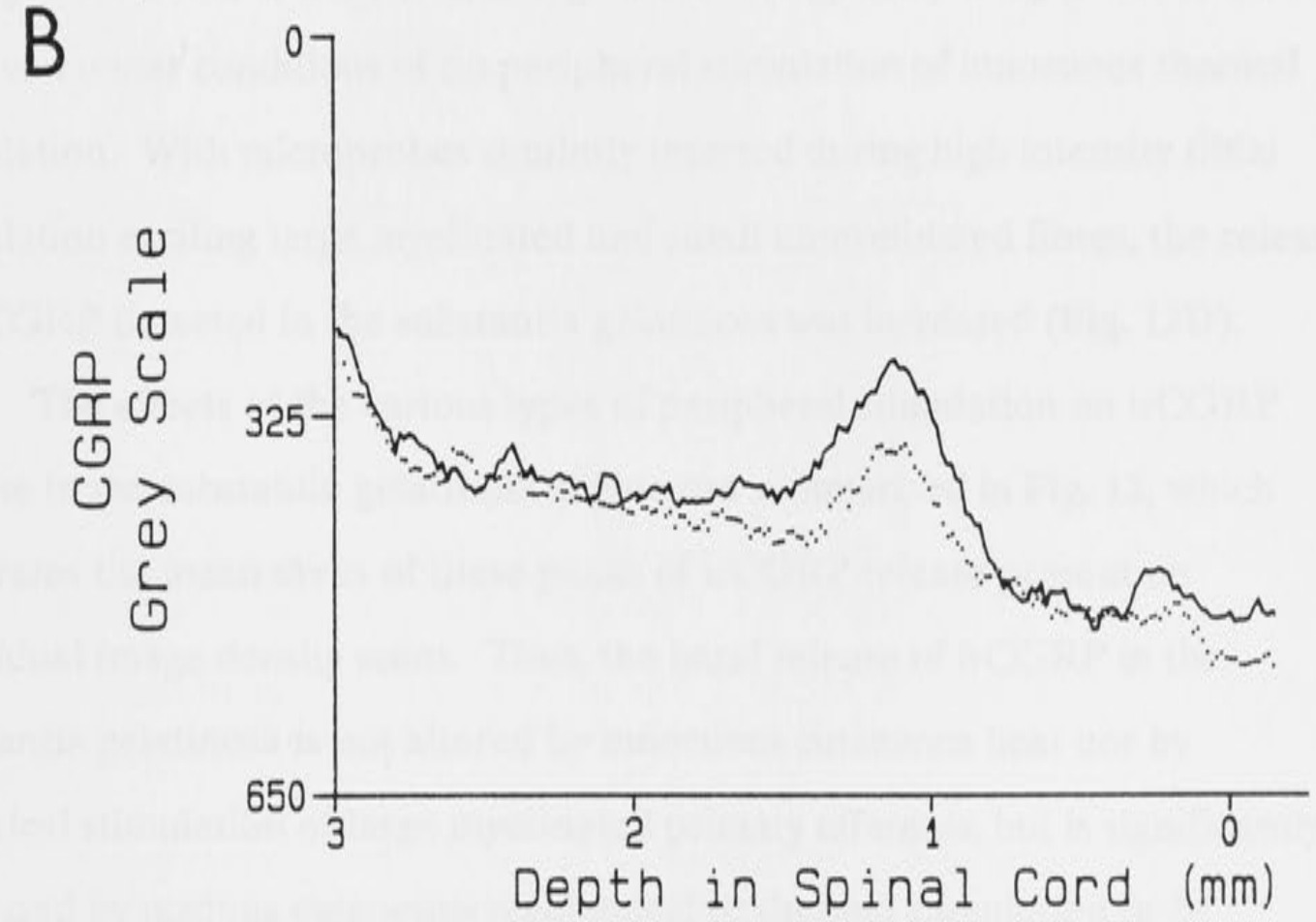
Fig. 11. Release of irCGRP in the lumbar dorsal horn.

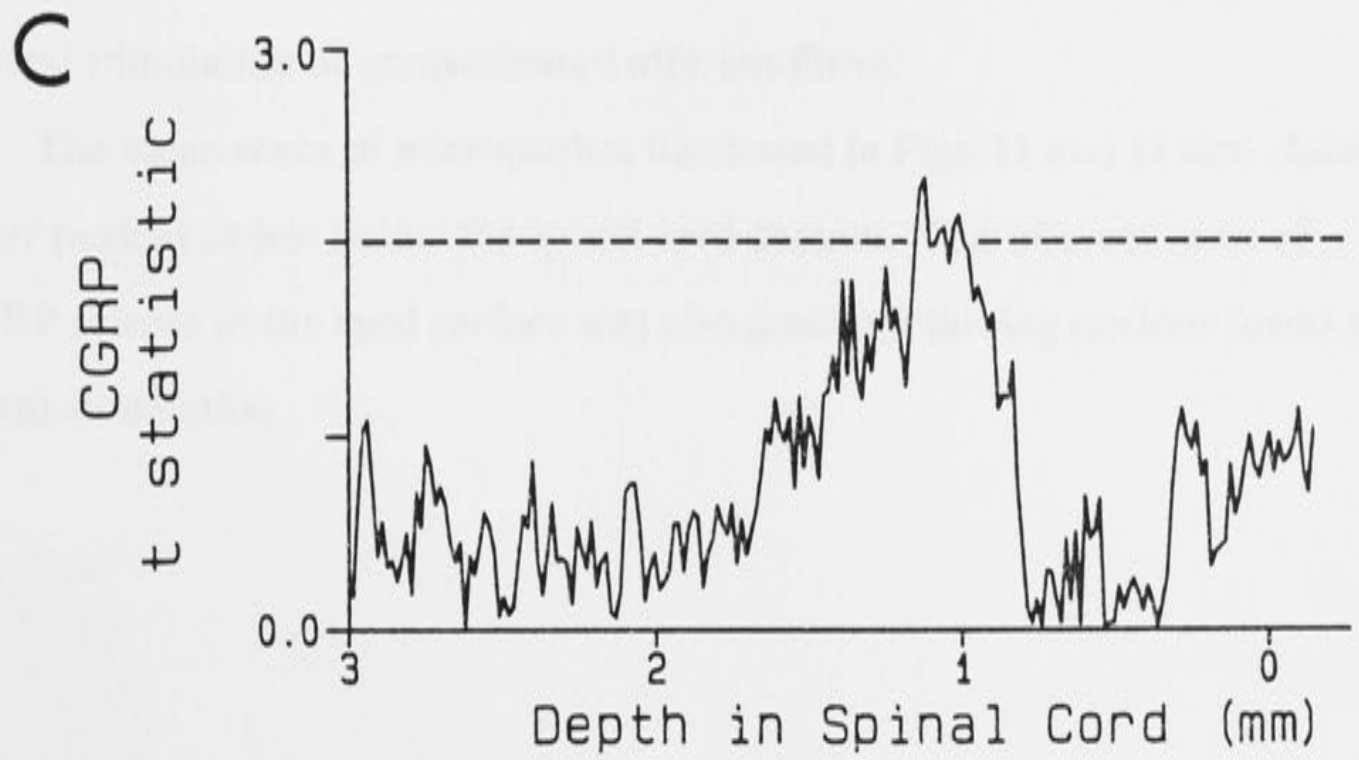
A The mean image density scan (and S.E.M.) of autoradiographs of 28 microprobes inserted into the spinal cord for 10 min in the absence of peripheral stimulation.

B The solid line is the mean scan of 27 microprobes inserted into the cord for 10 min during noxious mechanical stimulation of the ipsilateral hind paw. The broken line is mean scan A replotted. Standard errors have been omitted for clarity.

C The t-values calculated for the differences between the mean scans in B. The lower line is the t-value for significance at $P = 0.05$.


A
CGRP
Grey Scale
0
325
650
3
2
1
0
Depth in Spinal Cord (mm)


B
CGRP
Grey Scale
0
325
650
3
2
1
0
Depth in Spinal Cord (mm)


C
CGRP
t statistic
3.0
0.0
3
2
1
0
Depth in Spinal Cord (mm)

of the calculated t-values for the differences between the mean scans (**Fig. 11C**) shows that the peak of irCGRP release in the substantia gelatinosa region is significantly greater during noxious mechanical stimulation.

Release of irCGRP was measured during cutaneous thermal stimulation at either innocuous (41°C) or noxious 52°C) temperatures. With 41°C stimulation (**Fig. 12A**) irCGRP release was detected in the substantia gelatinosa region, similar to basal levels of release (**Fig. 11A**). When the thermal stimulus was noxious, however, this release of irCGRP was much greater (**Fig. 12B**).

Fig. 12C,D illustrated the mean scans of microprobes detecting irCGRP release during electrical stimulation of the ipsilateral tibial nerve. With low intensity stimulation exciting only the large myelinated fibres, irCGRP release was again detected in the substantia gelatinosa (**Fig. 12C**) comparable to that observed under conditions of no peripheral stimulation of innocuous thermal stimulation. With microprobes similarly inserted during high intensity tibial stimulation exciting large myelinated and small unmyelinated fibres, the release of irCGRP detected in the substantia gelatinosa was increased (**Fig. 12D**).

The effects of the various types of peripheral stimulation on irCGRP release in the substantia gelatinosa region are summarized in **Fig. 13,** which illustrates the mean areas of these peaks of irCGRP release present on individual image density scans. Thus, the basal release of irCGRP in the substantia gelatinosa is not altered by innocuous cutaneous heat nor by electrical stimulation of large myelinated primary afferents, but is significantly increased by noxious cutaneous mechanical or thermal stimulation or by electrical stimulation of unmyelinated afferent fibres.

The mean scans of microprobes illustrated in **Figs. 11** and **12** also show a smaller peak at or just below the spinal cord dorsum. This afferent zone of irCGRP release at the cord surface was also greater following noxious forms of afferent stimulation.

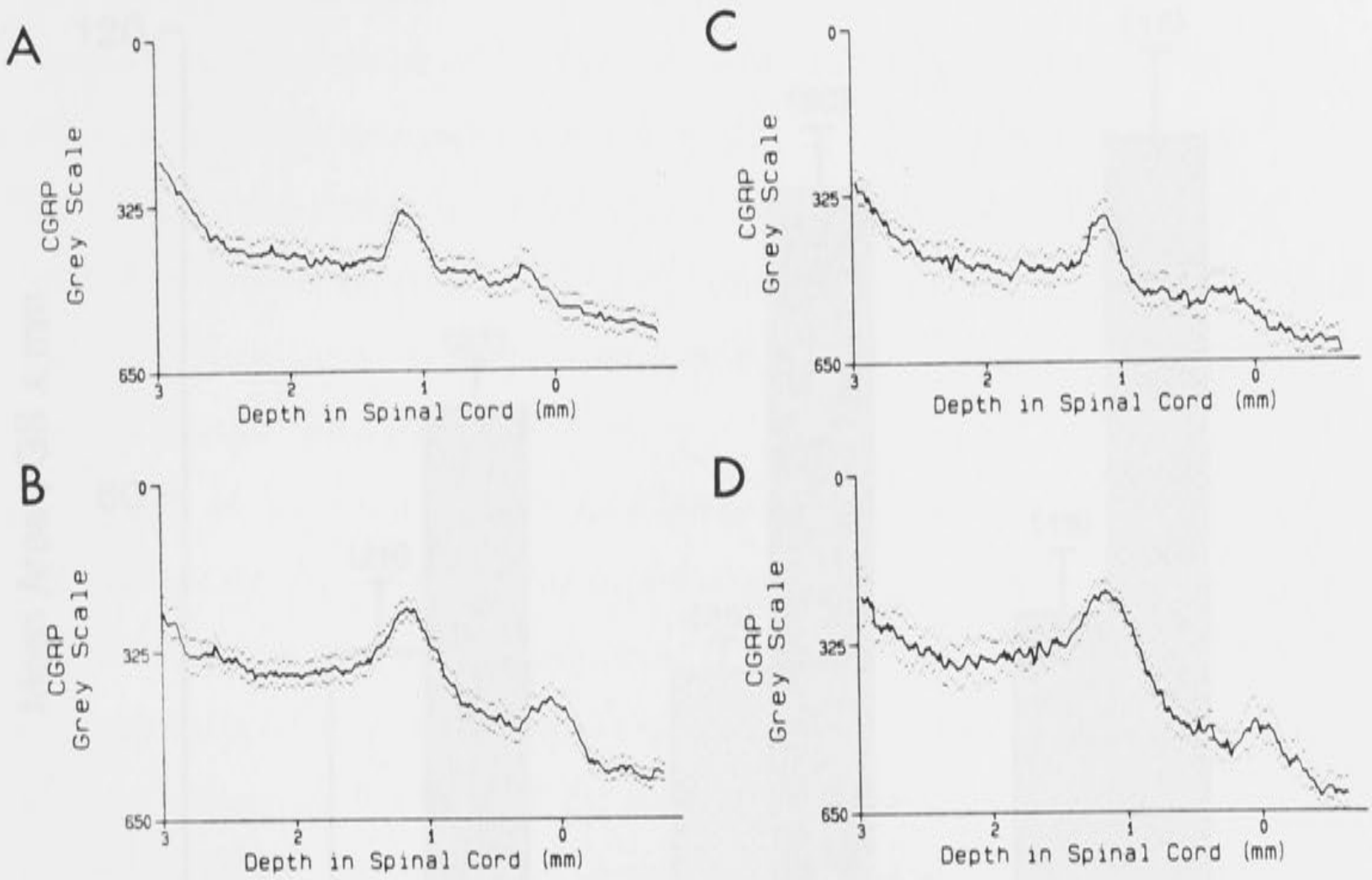


Fig. 12. Release of irCGRP in the dorsal horn with cutaneous thermal stimulation and with electrical stimulation of peripheral nerve.

A The mean image density scan (and S.E.M.) of 25 microprobes inserted into the lumbar cord for 10 min during immersion of the ipsilateral hind paw in water at 41^{o}C.

B The mean scan (and S.E.M.) of 30 microprobes similarly inserted for 10 min during immersion of the hind paw in water at 52^{o}C.

C The mean scan (and S.E.M.) of 18 microprobes inserted into the cord for 10 min during electrical stimulation of large myelinated afferents of the ipsilateral tibial nerve.

D The mean scan (and S.E.M.) of 15 microprobes similarly inserted for 10 min during tibial nerve stimulation sufficient to excite unmyelinated afferent fibres.

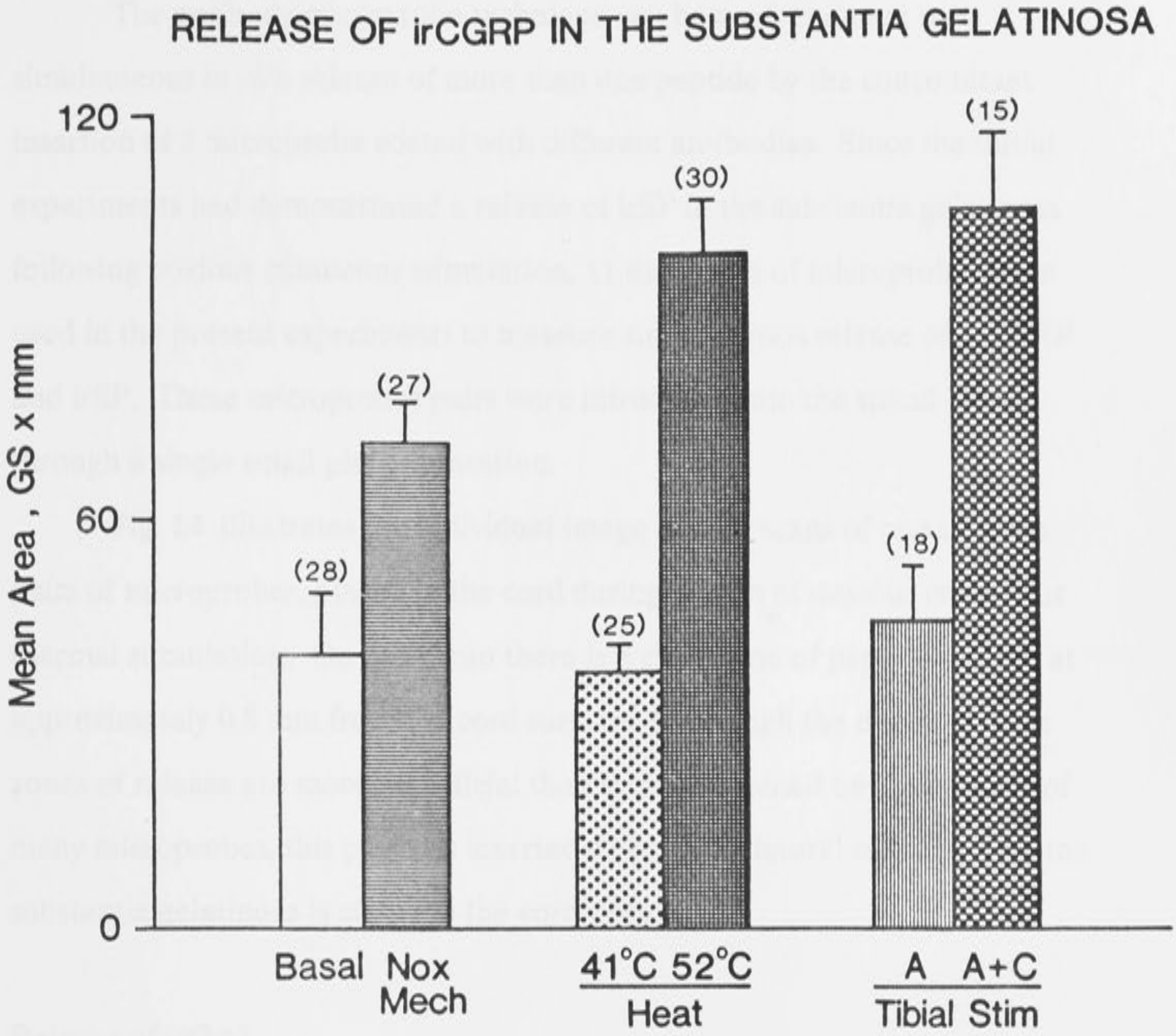


Fig. 13. Increased release of irCGRP in the substantia gelatinosa region with activation of nociceptive afferent fibres.
The histograms plot the mean area (and S.E.M.) under the peaks of irCGRP release in the substantia gelatinosa on individual image density scans of microprobes. The units of area are greyscale values x mm. The mean area on scans of microprobes inserted during noxious mechanical stimulation is significantly greater than the corresponding mean area representing basal irCGRP release ($0.001 < P < 0.01$). Similarly, the histograms representing irCGRP release during noxious thermal stimulation ($52^{o}C$) and electrical stimulation of both myelinated and unmyelinated tibial afferents (A + C) are significantly greater than their respective control histograms ($P < 0.001$). Numbers of microprobes in each group are shown in parentheses. Statistical comparisons were made by the Students t-test for unpaired data.

Simultaneous Release of irCGRP and irSP

The antibody microprobe technique can be used to detect the simultaneous *in vivo* release of more than one peptide by the concomitant insertion of 2 microprobe coated with different antibodies. Since the initial experiments had demonstrated a release of irSP in the substantia gelatinosa following noxious cutaneous stimulation, 11 such pairs of microprobes were used in the present experiments to measure simultaneous release of irCGRP and irSP. These microprobes pairs were introduced into the spinal cord through a single small pial preparation.

Fig. 14 illustrates the individual image density scans of one of these pairs of microprobes, placed in the cord during 10 min of noxious cutaneous thermal stimulation. On each scan there is a clear zone of peptide release at approximately 0.8 mm from the cord surface. (Although the depth of these zones of release are more superficial than that determined on mean scans of many microprobes, this pair was inserted close to the lateral sulcus, where the substantia gelatinosa is closer to the cord dorsum).

Release of irGAL

Basal release

An analysis of microprobes placed in the spinal cord in the absence of peripheral stimulation revealed a basal release of irGAL in the region of the substantia gelatinosa and at the spinal cord surface. The mean image density scans of such microprobes are illustrated in **Fig. 15.** With microprobes inserted for 10 min, there was a small zone of inhibition of binding just below the cord dorsum (**Fig. 15A**). Microprobe inserted for 30 min displayed not only this zone of surface release but additionally a second peak of release centered 1.1-1.2 mm from the cord surface (**Fig. 15B**). Thus, the sites of the peaks or irGAL release were comparable to those determined for irCGRP but the relative magnitude of the 2 peaks was reversed. Some microprobes inserted for 10 min showed a

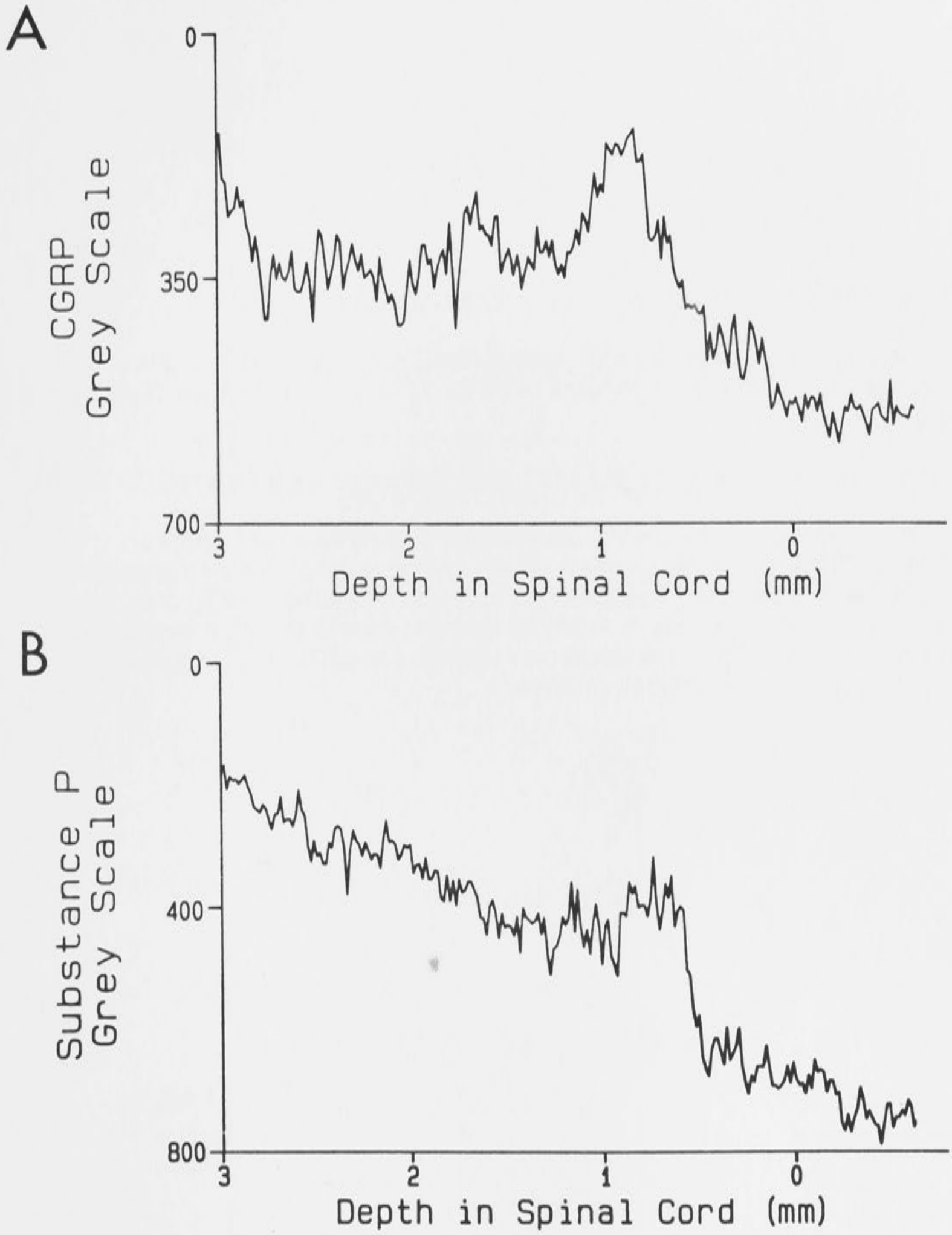


Fig. 14. Simultaneous release of irCGRP and irSP in the substantia gelatinosa.

A,B Image density scans of single microprobes detecting either irCGRP (A) or irSP (B) release. Both microprobes were concurrently placed in close apposition in the spinal cord through a single pial opening on the lateral dorsal cord surface, during immersion of the ipsilateral hind paw in water at 52°C.

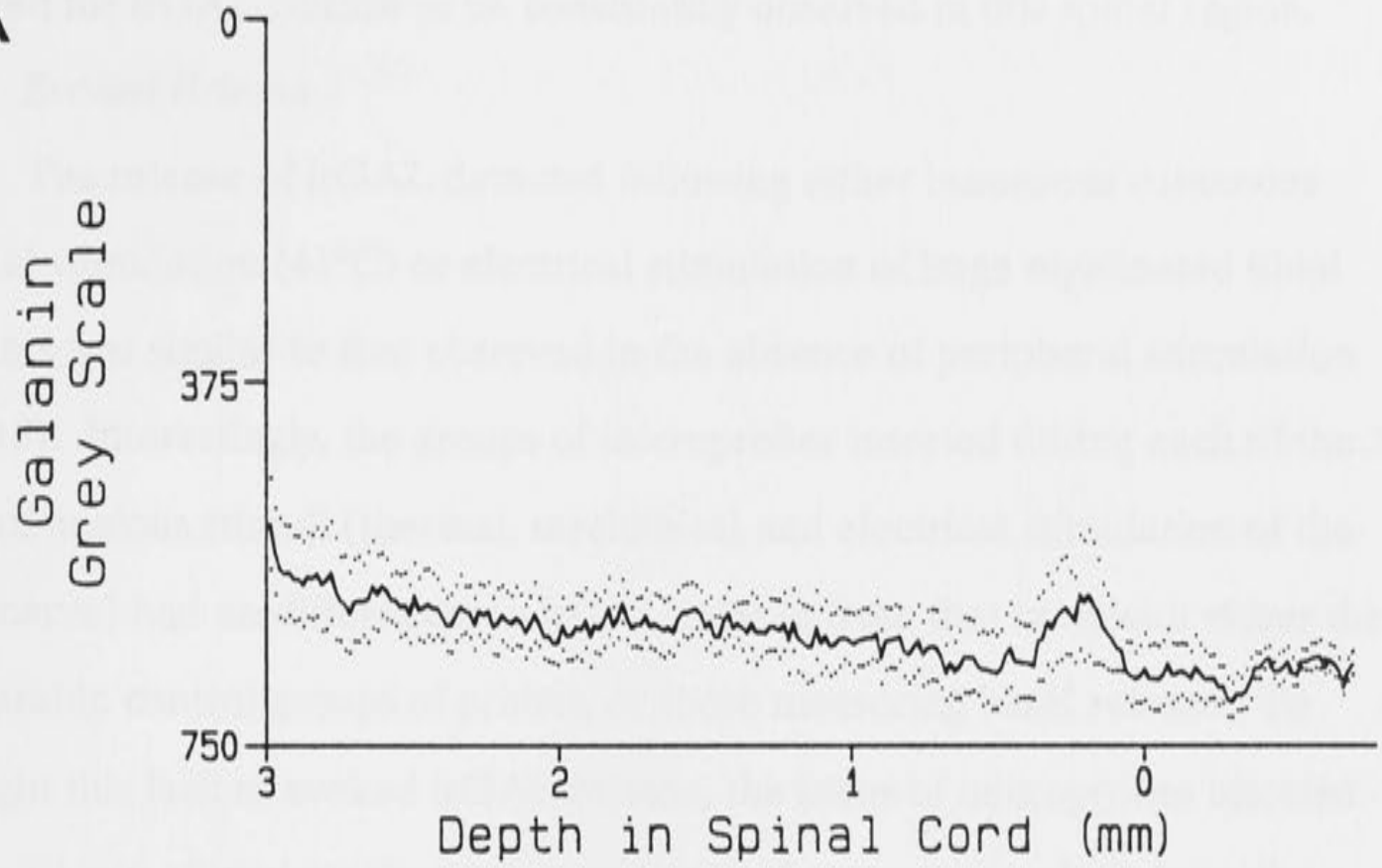
Fig. 15. Release of ir-galanin in the lumbar spinal cord.

A The mean image density scan (and S.E.M.) of the autoradiographs of 8 microprobes inserted into the spinal cord for 10 min in the absence of peripheral stimulation.

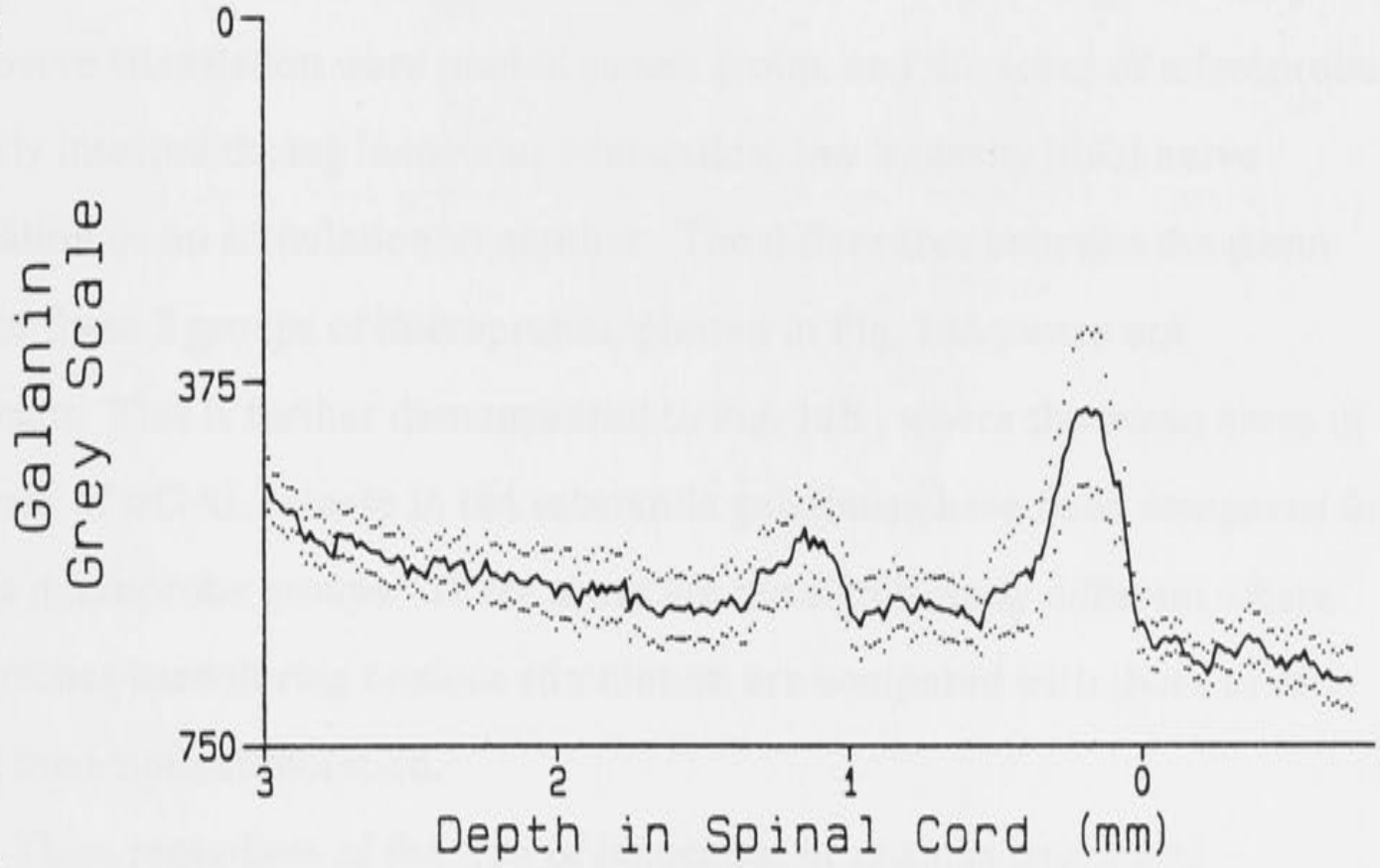
B The mean scan (and S.E.M.) of 11 microprobes similarly inserted for 30 min.

C The differences between the mean image density scan of microprobes (n = 28) inserted into the spinal cord for 30 min during noxious cutaneous stimulation or tibial nerve stimulation adequate to excite unmyelinated (C) afferents, and the mean scan of microprobes (n = 31) similarly inserted during non-noxious stimulation, tibial nerve stimulation only sufficient to excite large myelinated afferents, or in the absence of stimulation.

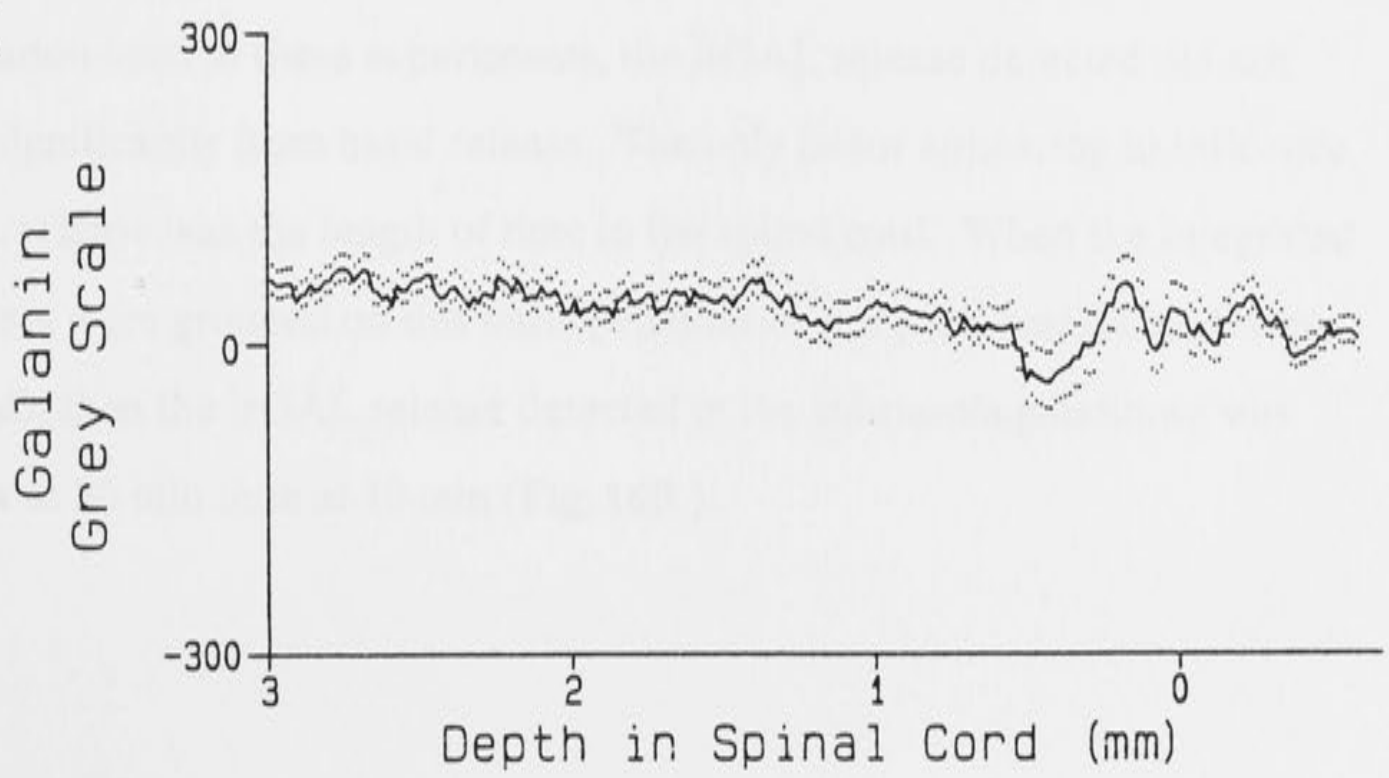
A


Galanin
Grey Scale
0
375
750
3
2
1
0
Depth in Spinal Cord (mm)

B


Galanin
Grey Scale
0
375
750
3
2
1
0
Depth in Spinal Cord (mm)

C


Galanin
Grey Scale
300
0
-300
3
2
1
0
Depth in Spinal Cord (mm)

small peak in the substantia gelatinosa but the longer time of insertion was required for irGAL release to be consistently observed in this spinal region.

Evoked Release

The release of irGAL detected following either innocuous cutaneous thermal stimulation (41°C) or electrical stimulation of large myelinated tibial afferents was similar to that observed in the absence of peripheral stimulation (**Fig. 15**). Interestingly, the groups of microprobes inserted during each of the 3 types of noxious stimuli (thermal, mechanical and electrical stimulation of the tibial nerve) had no differences in irGAL release from that seen with either the comparable control groups of probes, or those measuring basal release. To highlight this lack of evoked irGAL release, the scans of microprobes inserted during 30 min of noxious thermal or mechanical cutaneous or high intensity tibial nerve stimulation were pooled as one group, and the scans of microprobes similarly inserted during innocuous stimulation, low intensity tibial nerve stimulation or no stimulation as another. The differences between the mean scans of these 2 groups of microprobes, plotted in **Fig. 16A** , were not significant. This is further demonstrated in **Fig. 16B** , where the mean areas of the peaks of irGAL release in the substantia gelatinosa have been compared for various microprobe groups. These areas are not significantly different where microprobes used during noxious stimulation are compared with those used during innocuous stimulation.

Thus, regardless of the type of innocuous or noxious peripheral stimulation used in these experiments, the irGAL release detected did not differ significantly from basal release. The only factor appearing to influence irGAL release was the length of time in the spinal cord. When the integrated area data were grouped on this basis (whether or not peripheral stimuli were applied), then the irGAL release detected in the substantia gelatinosa was greater at 30 min than at 10 min (**Fig. 16B**).

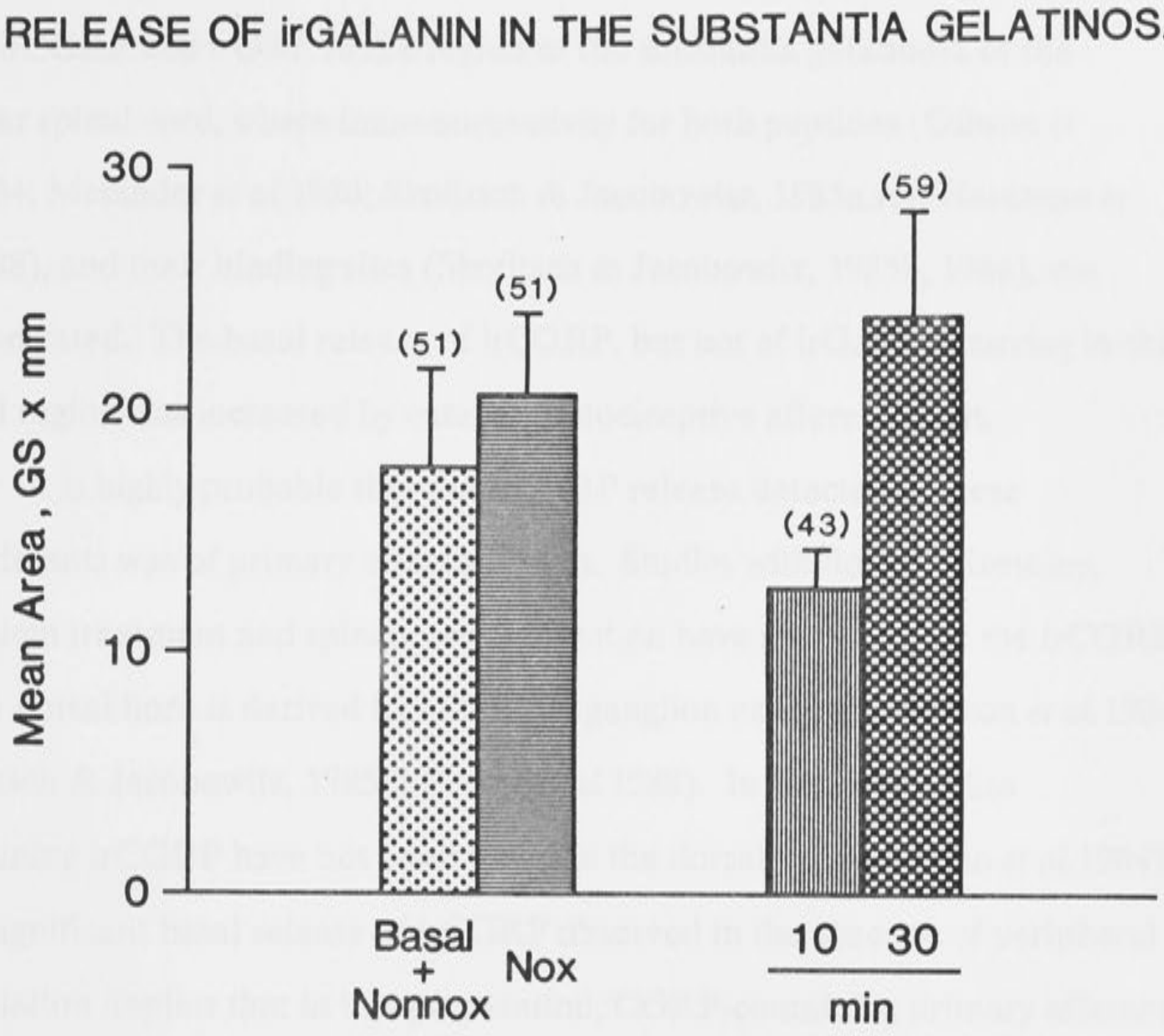


Fig. 16. Activation of nociceptive afferents does not increase ir-galanin release in the substantia gelatinosa.

The histograms plot the mean area (and S.E.M.) under the peaks of ir-galanin release in the substantia gelatinosa on image density scans of microprobes inserted in the spinal cord for 10 - 30 min. The units of area are greyscale values x mm. These mean areas are not significantly different on microprobes inserted during activation (cutaneous or tibial) of nociceptive afferents compared with those inserted during non-noxious (cutaneous or tibial) stimulation or no stimulation. The mean area on scans of microprobes inserted for 30 min (regardless of presence or type of peripheral stimulus) is significantly greater than the corresponding area on microprobes inserted for 10 min (also regardless of stimulus) ($0.01 < P < 0.05$). Numbers of microprobes are shown in parentheses. Statistical comparisons were made by the Students t-test for unpaired data.

Discussion

The experiments described in this section have detected a release of both irCGRP and irGAL in the region of the substantia gelatinosa of the lumbar spinal cord, where immunoreactivity for both peptides (Gibson *et al.* 1984; Melander *et al.* 1986; Skofitsch & Jacobowitz, 1985a,c,e; Harmann *et al.* 1988), and their binding sites (Skofitsch & Jacobowitz, 1985b, 1986), are concentrated. The basal release of irCGRP, but not of irGAL, occurring in this spinal region was increased by cutaneous nociceptive afferent input.

It is highly probable that the irCGRP release detected in these experiments was of primary afferent origin. Studies with dorsal rhizotomy, capsaicin treatment and spinal cord transection have revealed that the irCGRP in the dorsal horn is derived from sensory ganglion neurones (Gibson *et al.* 1984; Skofitsch & Jacobowitz, 1985d; Chung *et al.* 1988). In fact, cell bodies containing irCGRP have not been found in the dorsal horn (Gibson *et al.* 1984). The significant basal release of irCGRP observed in the absence of peripheral stimulation implies that in this preparation, CGRP-containing primary afferent fibres are tonically active. The peripheral source(s) of these afferent fibres releasing irCGRP are not certain but irCGRP has been found within a large number of primary sensory neurones of all sizes in several species including the cat (Gibson *et al.* 1984; Ju *et al.* 1987), and of cutaneous, muscular, and visceral origin (Molander *et al.* 1987; Gibbins *et al.* 1987). In addition to those afferents conveying non-nociceptive information from skin, muscle, and viscera, the lumbar cord segments studies would have also received continuous nociceptive input from areas subjected to the surgery required for the experiments.

The increased irCGRP release produced by the intentional activation of nociceptive afferents is in accord with immunohistochemical reports of irCGRP presence in a substantial proportion of small DRG neurones (Gibson *et al.* 1984; Ju *et al.* 1987). Since a variety of nociceptive stimuli were effective it is likely that this release occurred from the central terminals of polymodal nociceptors

(and possibly also specialized nociceptors). There are numerous reports of widespread coexistence of irCGRP and irSP within various sensory neurones in several species (Gibbins *et al.* 1985; Uddman *et al.* 1985; Lundberg *et al.* 1985; Lee *et al.* 1985b; Wanaka *et al.* 1986; Gazelius *et al.* 1987; Ju *et al.* 1987; Gibbins *et al.* 1987; Molander *et al.* 1987), even within the same secretory vesicles of these cells (Gulbenkian *et al.* 1986; Merighi *et al.* 1988). Importantly, many authors have noted that most if not all irSP-containing primary sensory neurones also contain irCGRP (Gibbins *et al.* 1985; Lee *et al.* 1985b; Lee *et al.* 1985a; Wanaka *et al.* 1986; Ju *et al.* 1987; Gazelius *et al.* 1987; Gibbins *et al.* 1987; Molander *et al.* 1987). Thus, not only would noxious peripheral stimuli previously shown to evoke irSP release in the substantia gelatinosa be likely to produce irCGRP release in the same area, but it is also probable that at least part of the simultaneous release of irSP and irCGRP detected in the present experiments was in fact co-release from the one subpopulation of neurones containing both these peptides. In addition, release of irCGRP from other primary afferents containing irCGRP but devoid of irSP could also have occurred.

The source of the irGAL release observed in the substantia gelatinosa is less certain. Primary afferents are one possibility: irGAL is found in some small capsaicin-sensitive sensory ganglion neurones and fibres containing irGAL are markedly depleted from the superficial spinal laminae by dorsal root section and capsaicin treatment (see **General Introduction**). However, irGAL has also been localised within intrinsic neurones of lamina I-II of the dorsal horn (Ch'ng *et al.* 1985; Melander *et al.* 1986) and a basal release from these cells could conceivably occur, with the relevant stimulus peripherally or centrally derived. Assuming irGAL release from primary afferents, the apparent inability of the peripheral stimulation procedures employed to increase this release raises the possibility of release from visceral rather than cutaneous or muscle afferents. In the rat, both irCGRP and irSP have been found in the subpopulation of small DRG neurones containing irGAL (Ju *et al.* 1987), and also in visceral

primary sensory neurones (Molander *et al.* 1987). The presence of irGAL in such afferents from viscera, however, has not yet been described. Although irGAL in the spinal cord may derive from supraspinal structures, it is not a possible source of galanin release observed since the cord was transected in these experiments.

A further consideration is that an unknown proportion of the basal release of peptides detected with the antibody microprobe technique may result from the rupture of neuronal elements containing peptides during microprobe insertion in the cord. While the antibody microprobe is smaller than other devices used to measure release of compounds *in vivo*, the increased sensitivity of the technique may still detect release of peptide by trauma to neurones.

An additional interesting finding of the present work is the apparent release of both irCGRP and irGAL at the spinal cord surface. A release of irSP and of ir-somatostatin at this location has also been observed in other chapters of the present work. Although the source of apparent release at the cord dorsum is not certain, it is possible that a local inflammatory response to the foreign proteins present on microprobes, or to minor trauma at sites of microprobe insertion, could evoke peptide release from pial afferent nerve endings. The occurrence of irCGRP in sensory nerves innervating the pia mater is well documented (Hanko *et al.* 1985; Uddman *et al.* 1985; Tsai *et al.* 1988), and the present results suggest that these nerves may also contain GAL. Several authors have speculated that vasoactive peptides in pial nerves could regulate blood flow to the central nervous system (reviewed in Edvinsson, 1985). The increased surface release observed with irCGRP (and previously with irSP) during noxious peripheral stimulation may be related to the finding that sensory neurones with nociceptive cutaneous receptive fields can also be activated by stimulation of the dura mater (Strassman *et al.* 1986). Thus, it is possible that a noxious cutaneous stimulus evokes peptide release at two discrete spinal sites.

The physiological functions of CGRP and GAL in the spinal cord are still conjectural. Administration of CGRP has produced excitation of neurones in the immature rat spinal cord *in vitro* (Ryu *et al.* 1988) and in the cat lumbar dorsal horn *in vivo* (Miletic & Tan, 1988), whereas GAL, on the other hand, has produced inhibitory effects on spinal reflexes *in vitro* (Yanagisawa *et al.* 1986), and in tests of analgesia (Post *et al.* 1988). However, although irCGRP is released during nociception, its widespread distribution in many sensory ganglion neurones of various sizes makes it an unlikely candidate for the transmission of a particular sensory modality, as discussed by others (Ju *et al.* 1987). Nevertheless, it is an attractive concept that the transmission of various types of noxious sensory information is accompanied by particular profiles of intraspinal release of several compounds. Evidence to date indicates that several forms of noxious cutaneous stimulation evokes release in the substantia gelatinosa of irSP (*vide supra*) and irCGRP, while ir somatostatin release additionally occurs with thermal noxious stimulation (*vide infra*). Moreover, it is possible that such release profiles may include glutamate which coexists with irSP in the central terminals of primary afferents (De Biasi & Rustioni, 1988). Thus it is becoming increasingly clear that the primary afferent transmission of nociceptive information may be a quite complex event, involving the simultaneous release and action of several compounds, amino acids and neuropeptides, on second order neurones.

Release of Somatostatin in the Dorsal Horn

Results

A total of 348 microprobes were analysed in this study. Of these, 103 microprobes were used *in vivo*, and 245 were used in *in vitro* tests.

Basal Release

To examine basal release of irSS, 46 microprobes were inserted into the spinal cord for periods of 5, 10, 15 or 30 min. On these microprobes there was a zone of release of irSS binding located 1.1-1.2 mm from the cord surface which, in the lower lumbar segments, corresponds to the region of lamina II, the substantia gelatinosa.

Fig. 17A illustrates the mean image density scan of 20 microprobes not exposed to SS prior to incubation in ^{125}I-SS. This mean scan thus represents the total binding of the labelled peptide (*in vitro* zero microprobes). **Fig. 17B** shows the mean image density scan for 16 microprobes inserted into the spinal cord for 5 min with no peripheral stimulation. Subtraction of the *in vitro* zero mean scan from that of the *in vivo* group gives the difference record in **Fig. 17C**. The difference was particularly significant in the region of the substantia gelatinosa (**Fig. 17D**).

Evoked Release

Tibial Nerve Stimulation

Previous experiments had demonstrated release of irSP in the region of the substantia gelatinosa in response to electrical stimulation of the tibial nerve at high (but not low) intensities. The present experiments therefore sought to examine the effect of such stimulation on irSS release. A total of 57 antibody microprobes was used for this analysis, inserted into the cord for 10 or 15 min.

Initially, a comparison was made between microprobes inserted during electrical stimulation of the ipsilateral tibial nerve at low intensities, exciting only the large myelinated fibres (1.5 x T for the most excitable fibres), and microprobes used to detect basal release (no nerve stimulation) in the same animals. In both the 10 and 15 min groups of probes, the low intensity tibial stimulation produced a small but statistically insignificant reduction in irSS

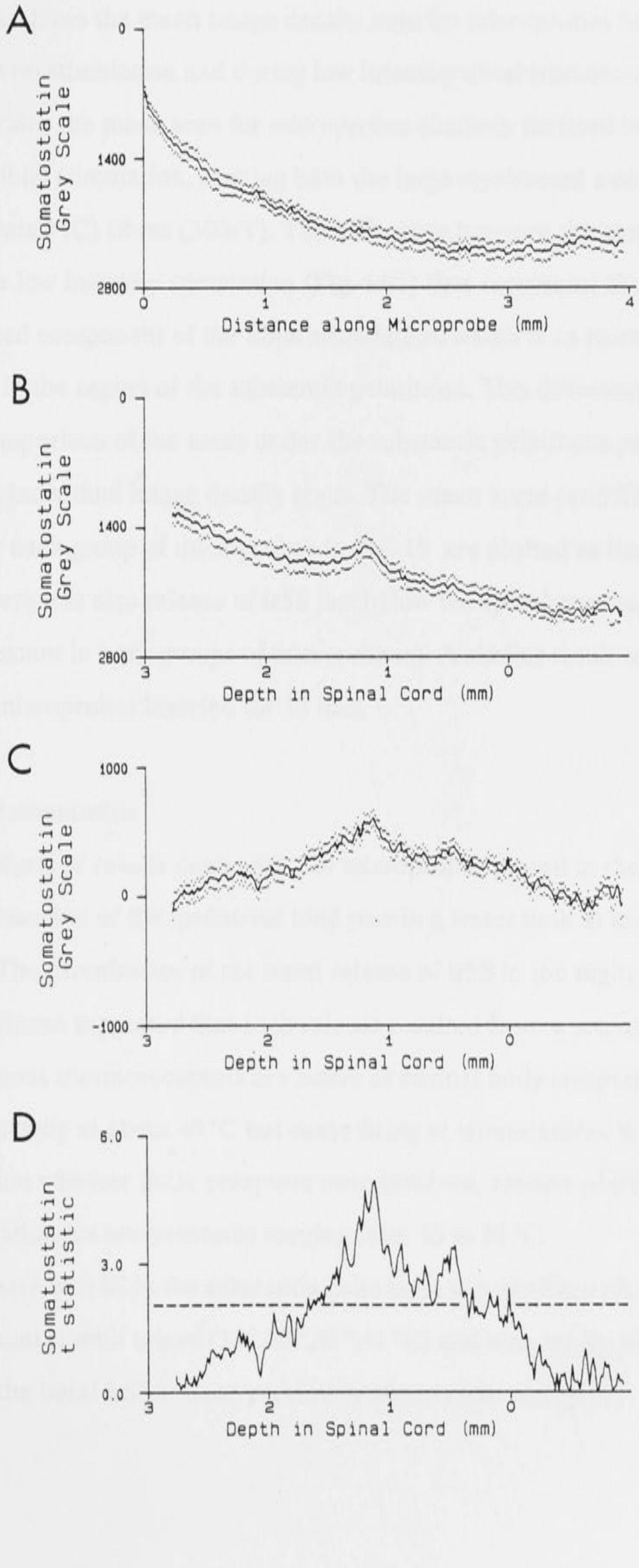
Fig. 17. Prominent basal release of irSS in the dorsal horn.

A: The mean image density scan (and SEM) of 20 microprobes incubated in ^{125}I-SS, plotted with respect to length.

B: The mean image density scan of 16 microprobes inserted 3 mm into the spinal cord for 5 min in the absence of nerve stimulation.

C: The differences between mean scans A and B.

D: The calculated t values for the differences of the means in C. The lower line is the t value indicating significance at P = 0.05.


A
Somatostatin
Grey Scale
0
1400
2800
0
1
2
3
4
Distance along Microprobe (mm)
B
Somatostatin
Grey Scale
0
1400
2800
3
2
1
0
Depth in Spinal Cord (mm)
C
Somatostatin
Grey Scale
1000
0
-1000
3
2
1
0
Depth in Spinal Cord (mm)
D
Somatostatin
t statistic
6.0
3.0
0.0
3
2
1
0
Depth in Spinal Cord (mm)

release in the substantia gelatinosa region. The data from the basal and the low intensity tibial stimulation groups of probes have therefore been pooled.

Fig. 18A shows the mean image density scan for microprobes inserted for 10 min with no stimulation and during low intensity tibial stimulation. **Fig. 18B** illustrates the mean scan for microprobes similarly inserted but with high intensity tibial stimulation, exciting both the large myelinated and the small unmyelinated (C) fibres (300xT). The difference between the mean scans for high and for low intensity stimulation (**Fig. 18C**) thus represents the effect of the unmyelinated component of the tibial stimulation, which is an increased release of irSS in the region of the substantia gelatinosa. This difference is also apparent by comparison of the areas under the substantia gelatinosa peaks of irSS release on individual image density scans. The mean areas (and SEM) of these peaks for each group of microprobes in **Fig. 18** are plotted as histograms in **Fig. 19.** (There was also release of irSS just below the spinal cord surface to a comparable extent in both groups of microprobes). A similar result was obtained with microprobes inserted for 15 min.

Thermal stimulation

This analysis of results deals with 124 microprobes placed in the spinal cord during immersion of the ipsilateral hind paw in a water bath at known temperatures. The prominence of the basal release of irSS in the region of the substantia gelatinosa suggested that such release resulted from a tonically active process. Cutaneous thermoreceptors are active at normal body temperature with maximal activity at about 43 °C but cease firing at temperatures below 28°C. To examine whether these receptors were involved, release of irSS was measured with stimulus temperatures ranging from 15 to 52 °C.

The release of irSS in the substantia gelatinosa was similar with all innocuous thermal stimuli tested (15 °,23 °,35 °,41 °C) and was not significantly different from the basal irSS release previously observed in this spinal

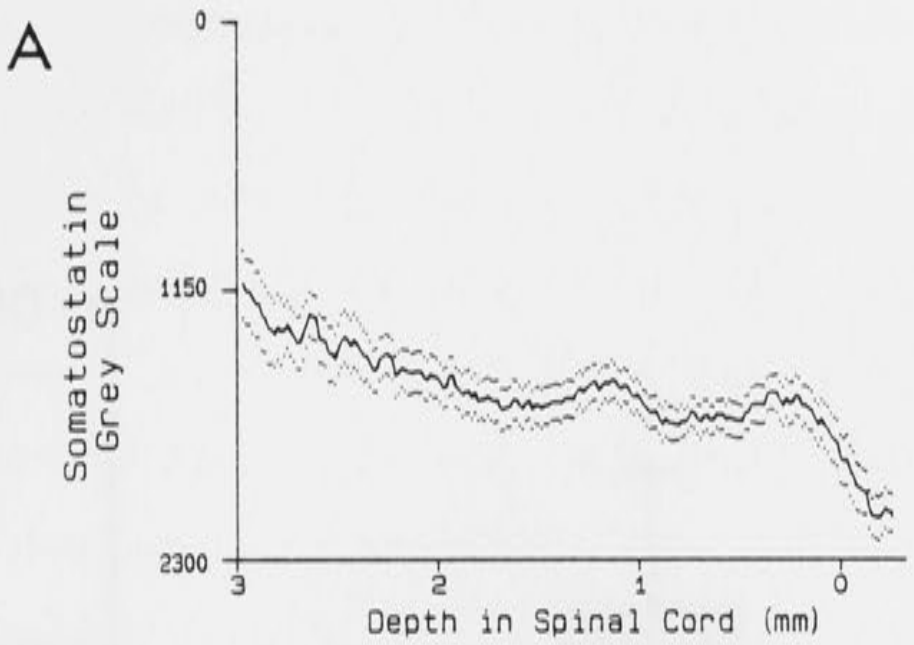
Fig. 18. Release of irSS with tibial nerve stimulation.

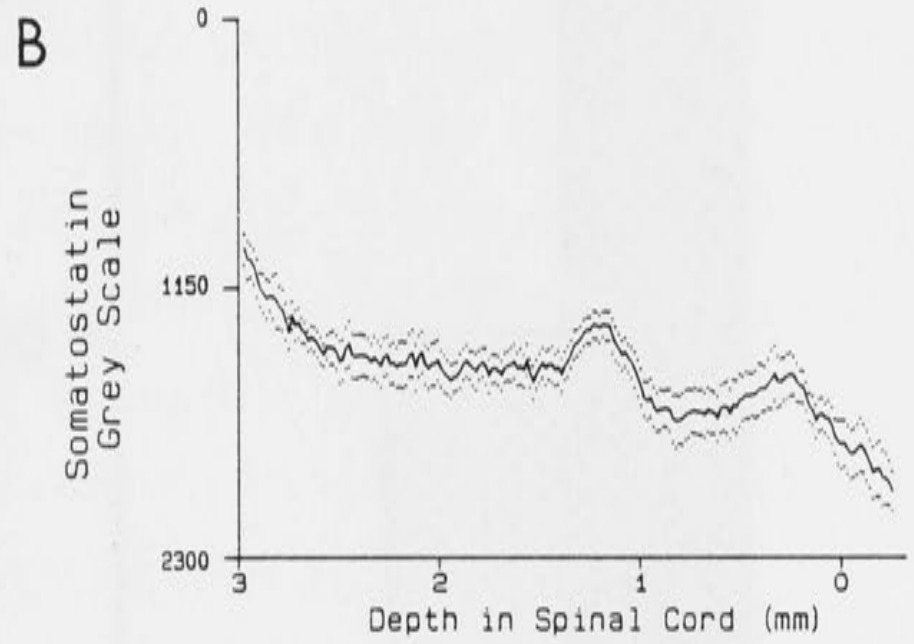
A. The mean image density scan of 19 microprobes inserted 3 mm into the spinal cord for 10 min during electrical stimulation of large myelinated afferents of the ipsilateral tibial nerve, or in the absence of stimulation.

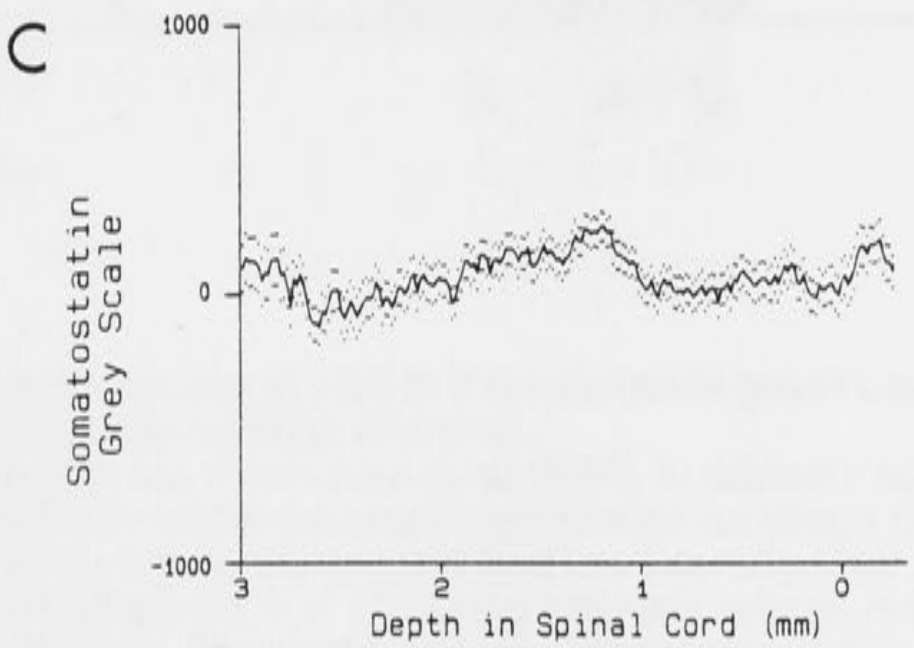
B: The mean image density scan of 12 microprobes inserted as in A, during tibial stimulation adequate to excite unmyelinated afferents.

C: The differences between the mean scans A and B.

D: The t values for the differences of the means. The lower line is the t value for significance at $P = 0.05$.


A
Somatostatin
Grey Scale
0
1150
2300
3
2
1
0
Depth in Spinal Cord (mm)


B
Somatostatin
Grey Scale
0
1150
2300
3
2
1
0
Depth in Spinal Cord (mm)


C
Somatostatin
Grey Scale
1000
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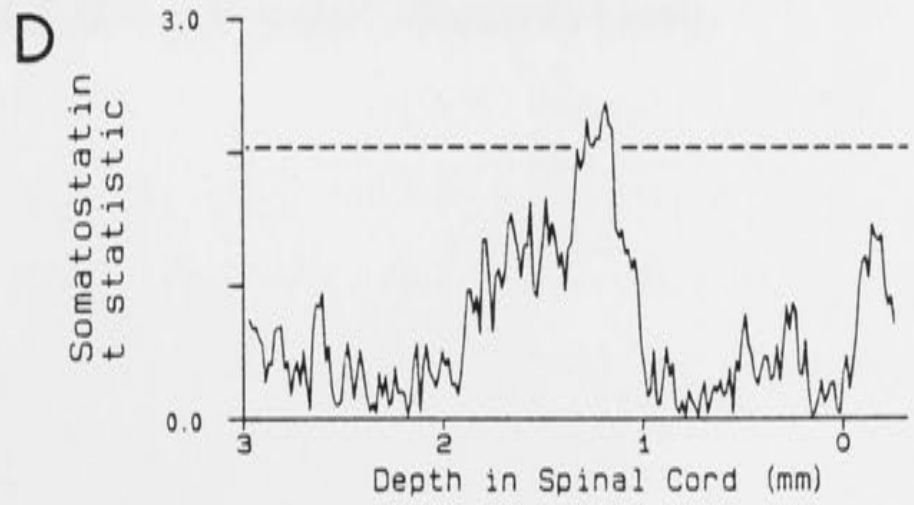
Depth in Spinal Cord (mm)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Depth in Spinal Cord (mm)

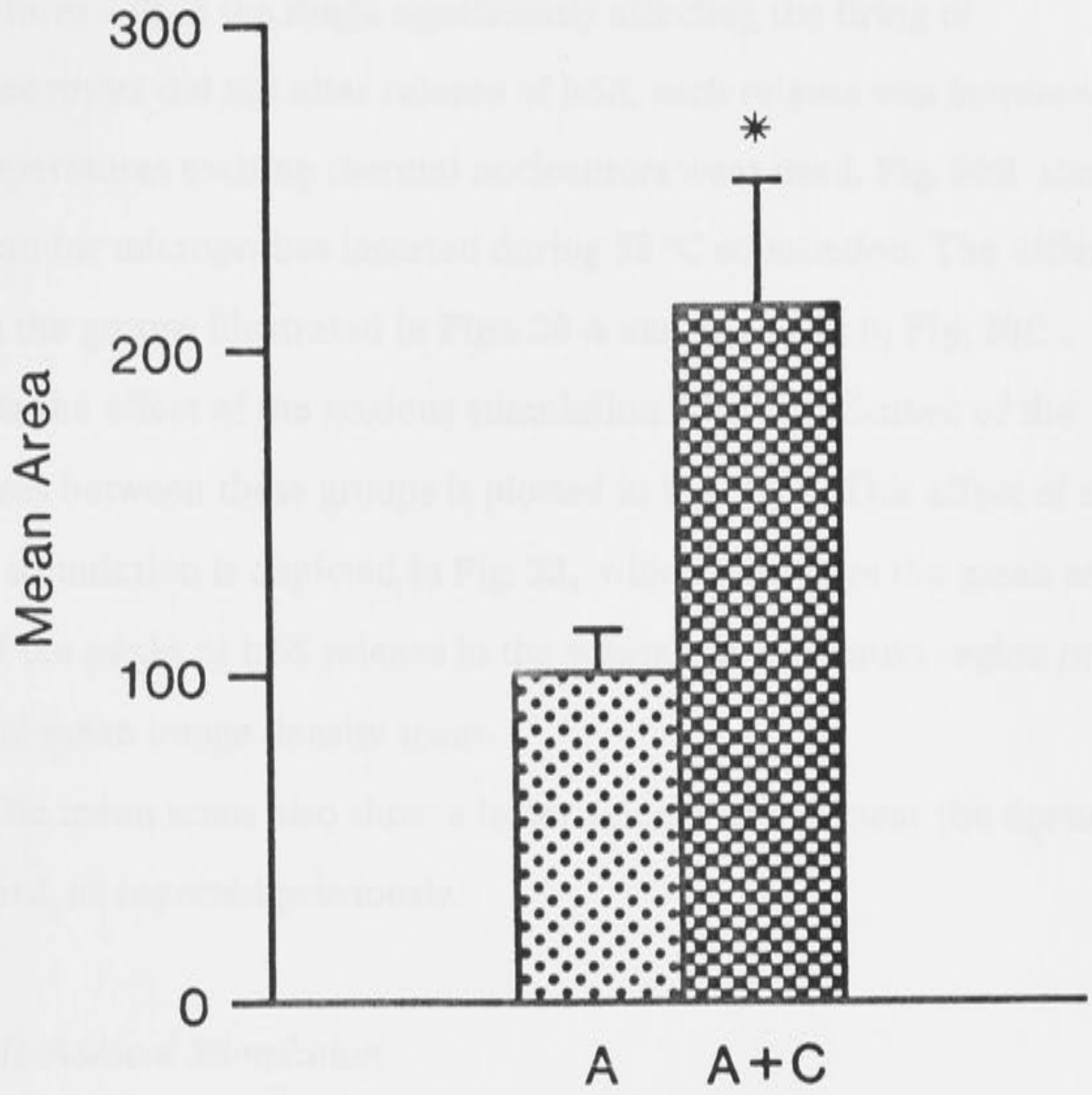


Fig. 19. Increased release of irSS in the substantia gelatinosa region with activation of unmyelinated tibial afferents.
The histograms plot the mean area (and SEM), in arbitrary square units, of the peaks of irSS release in the substantia gelatinosa on image density scans of microprobes. A, during no stimulation and low intensity tibial stimulation exciting large myelinated afferents; A + C, during high intensity stimulation exciting both A and C fibre afferents. The mean area is significantly greater when C fibres were activated ($0.001 < P < 0.01$, Students t-test).

region. **Fig. 20A** shows the mean image density scan of microprobes inserted in the spinal cord for 5 min during peripheral thermal stimulation at 41 °C. There is a peak on the scan at 1.1-1.2 mm from the cord surface, representing irSS release in the region of the substantia gelatinosa. Although varying stimulus temperatures within the range significantly affecting the firing of thermoreceptors did not alter release of irSS, such release was increased when skin temperatures exciting thermal nociceptors were used. **Fig. 20B** shows the mean scan for microprobes inserted during 52 °C stimulation. The difference between the groups illustrated in **Figs. 20 A** and **B**, shown in **Fig. 20C** , highlights the effect of the noxious stimulation. The significance of the differences between these groups is plotted in **Fig. 20D** . This effect of noxious thermal stimulation is depicted in **Fig. 22,** which illustrates the mean areas (and SEM) of the peaks of irSS release in the substantia gelatinosa region present on individual mean image density scans.

The mean scans also show a lesser release of irSS near the dorsum of the spinal cord, as reported previously.

Mechanical Stimulation

For this study 66 microprobes were analysed. Of these, 32 were inserted in the spinal cord during innocuous mechanical stimulation, and 34 during noxious mechanical stimulation.

The mean image density scan for microprobes inserted for 5 min during innocuous mechanical stimulation featured a peak of irSS release in the region of the substantia gelatinosa similar to that observed with innocuous thermal stimulation (**Fig. 21A**). This irSS release was not increased by noxious mechanical stimulation (**Fig. 21B**). **Fig. 21C** illustrates the difference between the mean scans for noxious and innocuous mechanical stimulation, and the t-statistic record (**Fig. 21D**) shows no difference in irSS release between these 2 groups. This is further demonstrated in **Fig. 22** which plots the mean areas (and

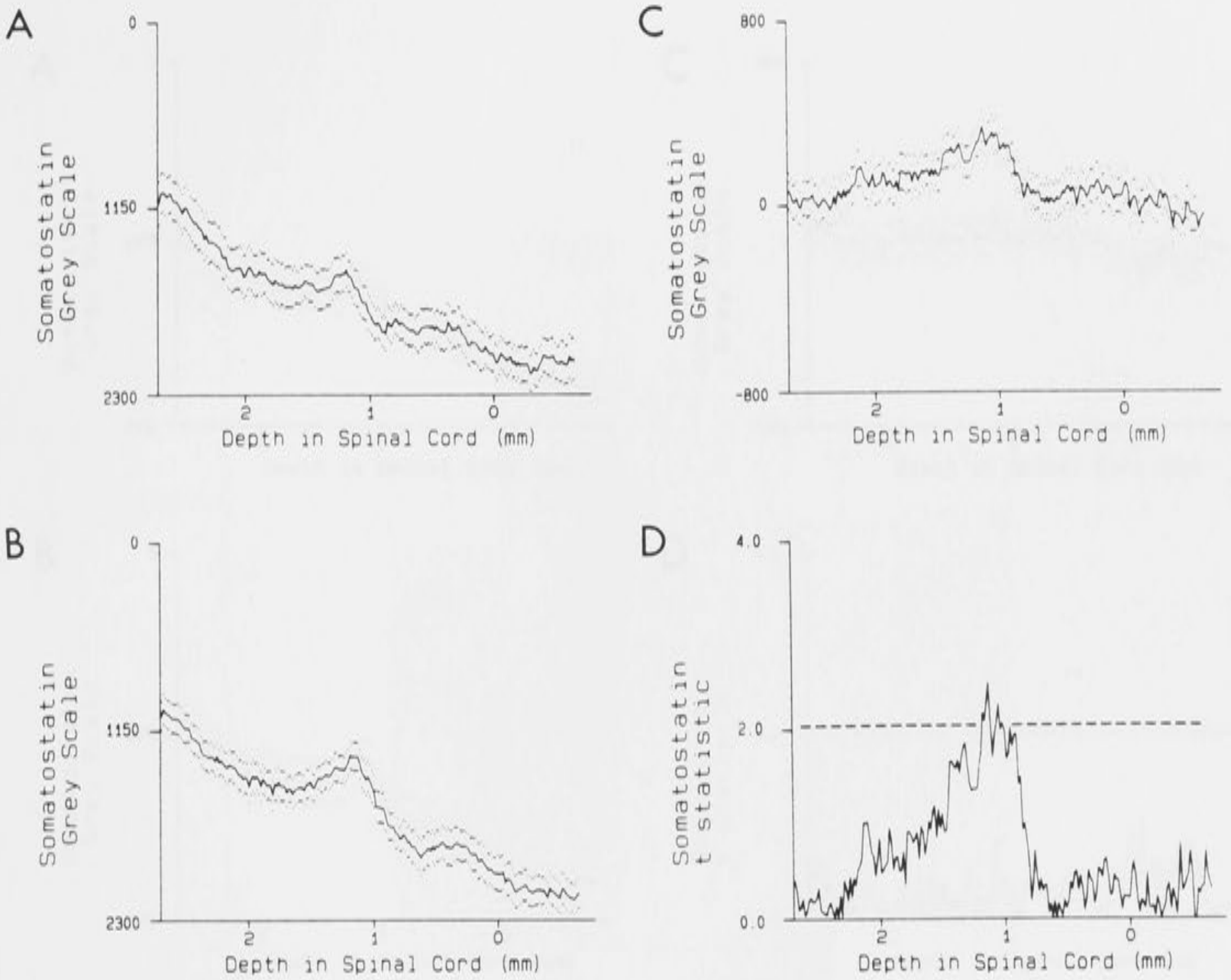


Fig. 20. Release of irSS in the dorsal horn with cutaneous thermal stimulation.

A: The mean image density scan (and SEM) of 14 microprobes inserted into the lower lumbar spinal cord for 5 min during immersion of the ipsilateral hind paw in water at 41° C.

B: The mean image density scan (and SEM) of 15 microprobes inserted into the spinal cord for 5 min during immersion of the hind paw in water at 52° C.

C: The differences between the mean scans plotted in A and B.

D: The t values calculated for the differences of the means.

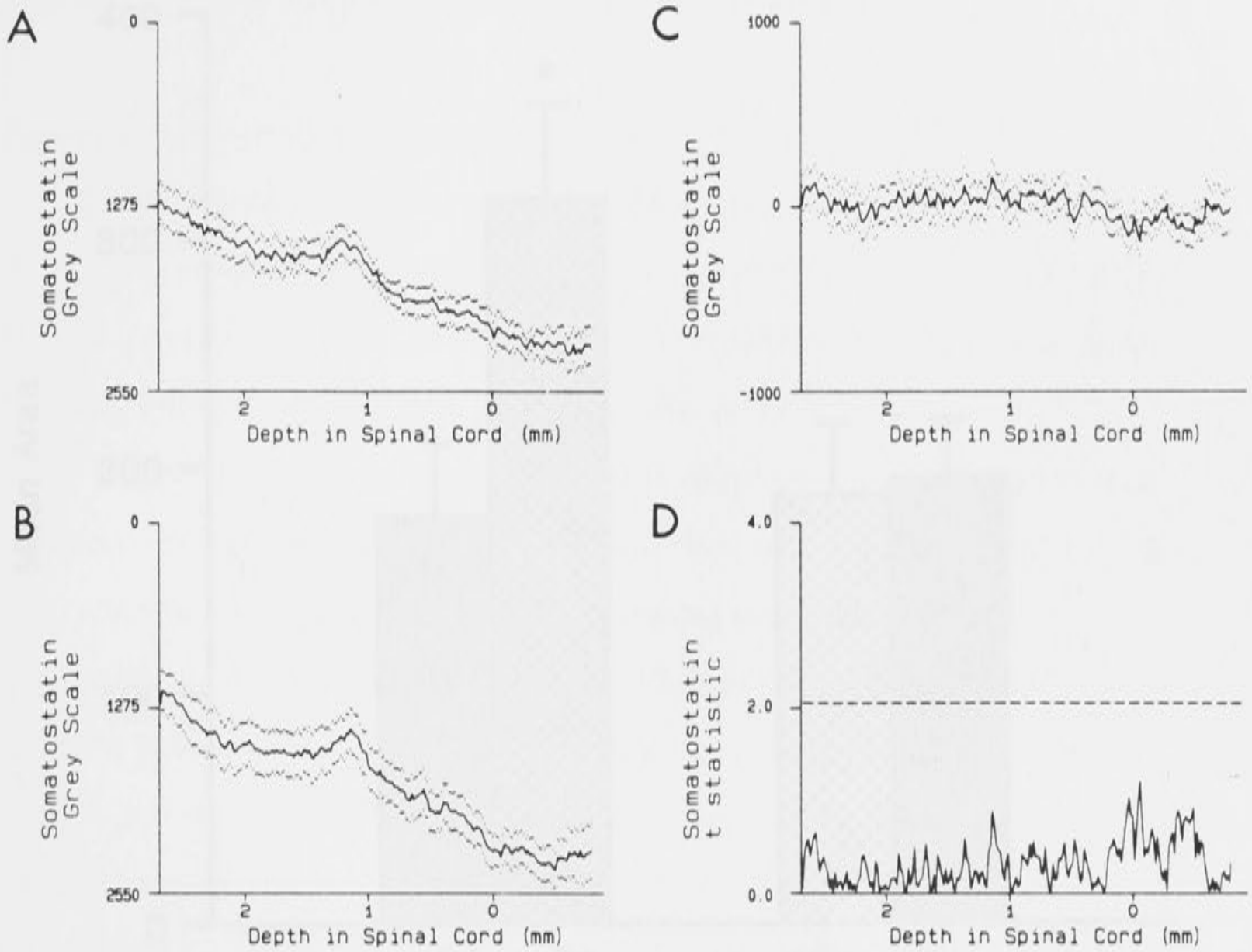


Fig. 21. Lack of release of irSS in the dorsal horn with cutaneous mechanical stimulation.

A: The mean image density scan (and SEM) of 13 microprobes inserted into the lower lumbar spinal cord for 5 min during innocuous mechanical stimulation of the ipsilateral hind paw.

B: The mean image density scan (and SEM) of 14 microprobes inserted into the spinal cord for 5 min during noxious mechanical stimulation of the hind paw.

C: The differences between the mean scans plotted in A and B.

D: The t values calculated for the differences of the means.

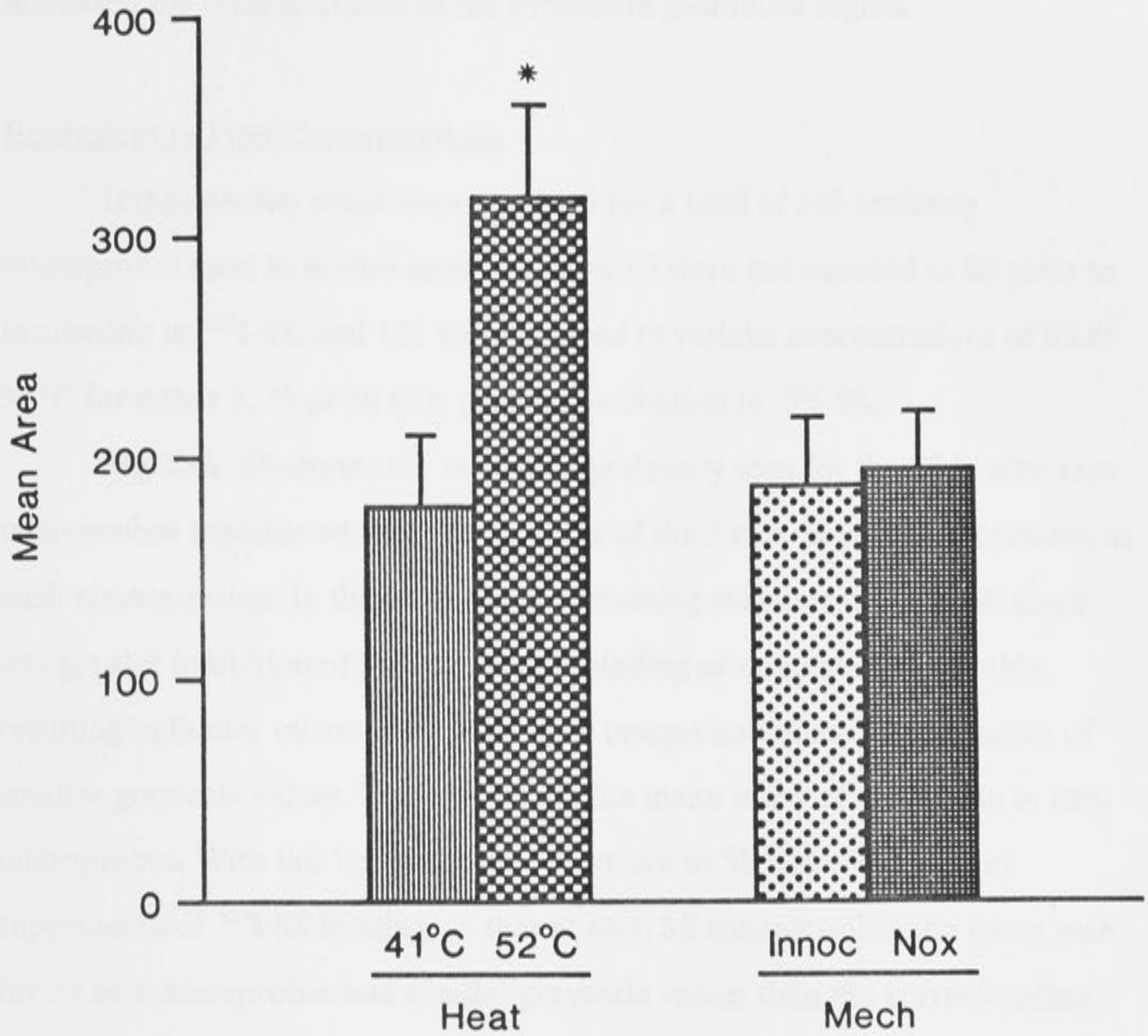


Fig. 22. Increased release of irSS in the substantia gelatinosa region with noxious thermal stimulation, but not with noxious mechanical stimulation nor innocuous forms of stimulation. The histograms plot the mean area (and SEM), in arbitrary square units, of the peaks of irSS release in the substantia gelatinosa on image density scans. The mean area on scans of microprobes inserted during noxious skin heating (52° C) is significantly greater than the corresponding mean area during innocuous skin heating (41° C) ($0.01 < P < 0.05$, Students t-test).

SEM) of the peaks of irSS release in the substantia gelatinosa, for both types of mechanical stimulation.

Thus, of the cutaneous stimuli tested, only noxious thermal stimulation increased the release of irSS in the substantia gelatinosa region.

Equivalent *In Vitro* Concentrations

Image density scans were obtained for a total of 245 antibody microprobes used in *in vitro* tests. Of these, 60 were not exposed to SS prior to incubation in ^{125}I-SS, and 185 were exposed to various concentrations of SS at 37 °C for either 5, 15 or 30 min, prior to incubation in ^{125}I-SS.

Fig. 23A illustrates the mean image density scan for the 60 *in vitro* zero microprobes together with the mean scans of the 5 min. *in vitro* microprobes, at each concentration. In the presence of increasing concentrations of SS there was greater inhibition of the subsequent binding of radiolabelled peptide, resulting in fainter microprobe X-ray film images and image density scans of smaller greyscale values. **Fig. 23B** shows the mean scans of the 30 min *in vitro* microprobes. With this longer time of exposure to SS there was greater suppression of ^{125}I-SS binding, so that at each SS concentration the mean scan for 30 min microprobes had smaller greyscale values than the corresponding scan for 5 min microprobes. A mean image density scan of unexposed areas of the X-ray films from these experiments (film background) has also been plotted.

The mean image density scans of *in vitro* microprobes were used to estimate the equivalent *in vitro* concentration of irSS in the region of the substantia gelatinosa. This was done by obtaining the ratio of the greyscale of an *in vivo* group of microprobes to that of the *in vitro* zero group. In **Fig. 24,** the greyscale of each 5 min *in vitro* microprobe was divided by the mean greyscale of the *in vitro* zero group of microprobes, at corresponding 16 μm steps along the length of the probes. The mean scans for each concentration were

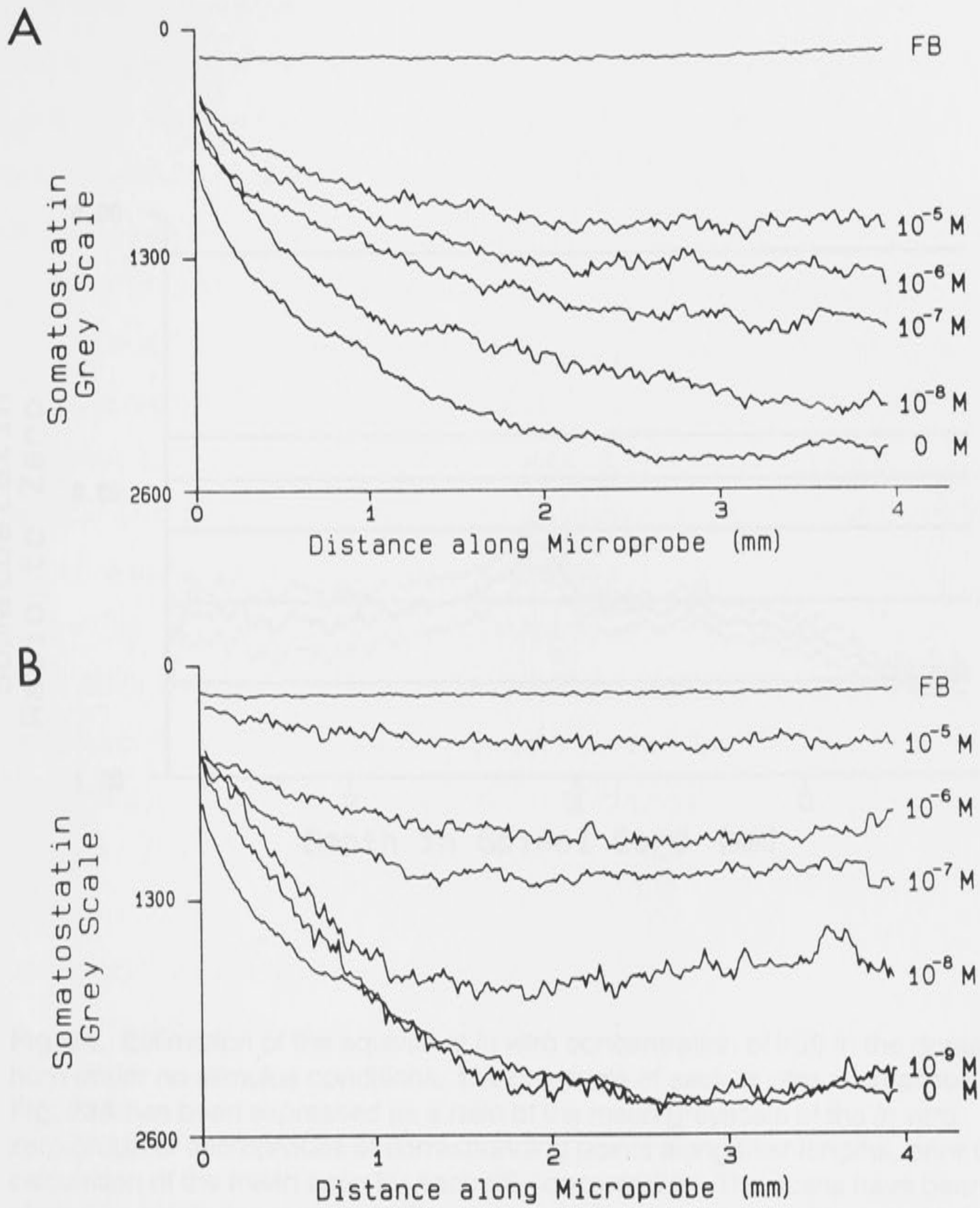


Fig. 23. Mean image density scans of *in vitro* microprobes. The mean scans of microprobes exposed to particular concentrations of SS *in vitro* for 5 min (**A**) or 30 min (**B**) prior to incubation in ^{125}I-SS are plotted with respect to length. The O M mean scan is of microprobes not exposed to SS prior to incubation in ^{125}I-SS (*in vitro* zeros). The standard errors of the scans have been omitted for clarity. FB, film background

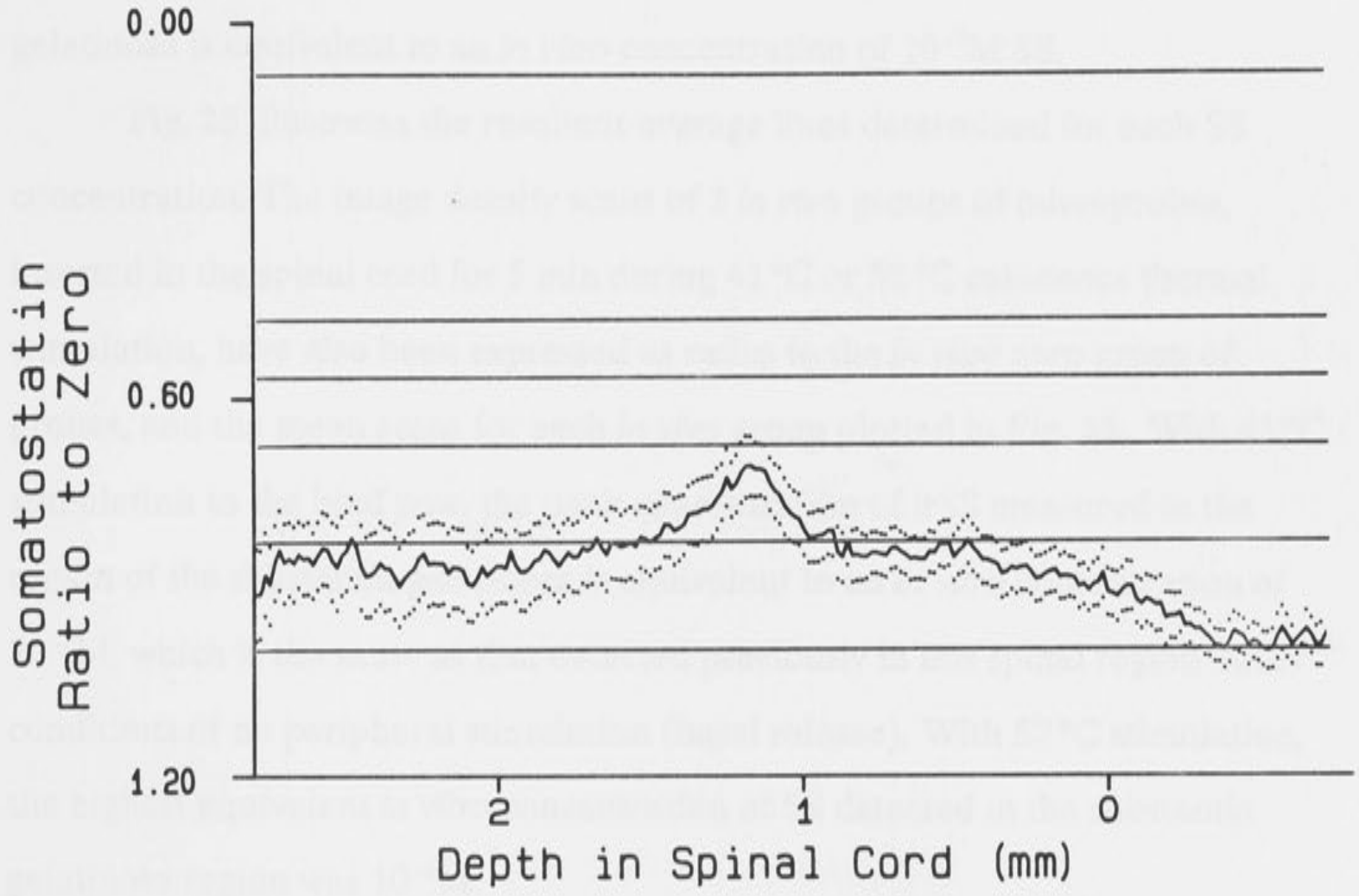


Fig. 24. Estimation of the equivalent *in vitro* concentration of irSS in the dorsal horn under no stimulus conditions. The greyscale of each *in vitro* microprobe in **Fig. 23A** has been expressed as a ratio of the mean greyscale of the *in vitro* zero group of microprobes at corresponding points along their lengths, prior to calculation of the mean scan for each SS concentration. The scans have been plotted as single line averages. The scans of microprobes detecting basal irSS release during 5 min of insertion in the spinal cord (**Fig. 23B**) have similarly been expressed as a ratio of the *in vitro* zero group. FB, film background.

were then calculated and the overall average ratio plotted as a single line. The image density scans of the microprobes inserted in the cord for 5 min. in the absence of peripheral stimulation were similarly converted to ratios to the *in vitro* zero group. It can be seen that under these *in vivo* conditions of basal irSS release, the concentration of irSS detected in the region of the substantia gelatinosa is equivalent to an *in vitro* concentration of 10^{-7}M SS.

Fig. 25 illustrates the resultant average lines determined for each SS concentration. The image density scans of 2 *in vivo* groups of microprobes, inserted in the spinal cord for 5 min during 41 °C or 52 °C cutaneous thermal stimulation, have also been expressed as ratios to the *in vitro* zero group of probes, and the mean scans for each *in vivo* group plotted in **Fig. 25.** With 41 °C stimulation to the hind paw, the peak concentration of irSS measured in the region of the substantia gelatinosa is equivalent to an *in vitro* concentration of 10^{-7}M, which is the same as that detected previously in this spinal region under conditions of no peripheral stimulation (basal release). With 52 °C stimulation, the highest equivalent *in vitro* concentration of SS detected in the substantia gelatinosa region was 10^{-6}M.

Simultaneous Release of irSS and irSP

Since the present experiments showed intraspinal release of irSS with noxious heating of the skin, a stimulus which also evokes irSP release (*vide supra*), this form of stimulation was used to detect possible simultaneous release of both peptides in the dorsal horn. This was done by the concomitant insertion of 2 antibody microprobes, one prepared to detect irSS and the other, irSP. These microprobe pairs were inserted into the same area of spinal cord through the one small opening in the pia mater during a noxious cutaneous heat stimulus. Thirty-seven such pairs of microprobes were used to measure simultaneous release.

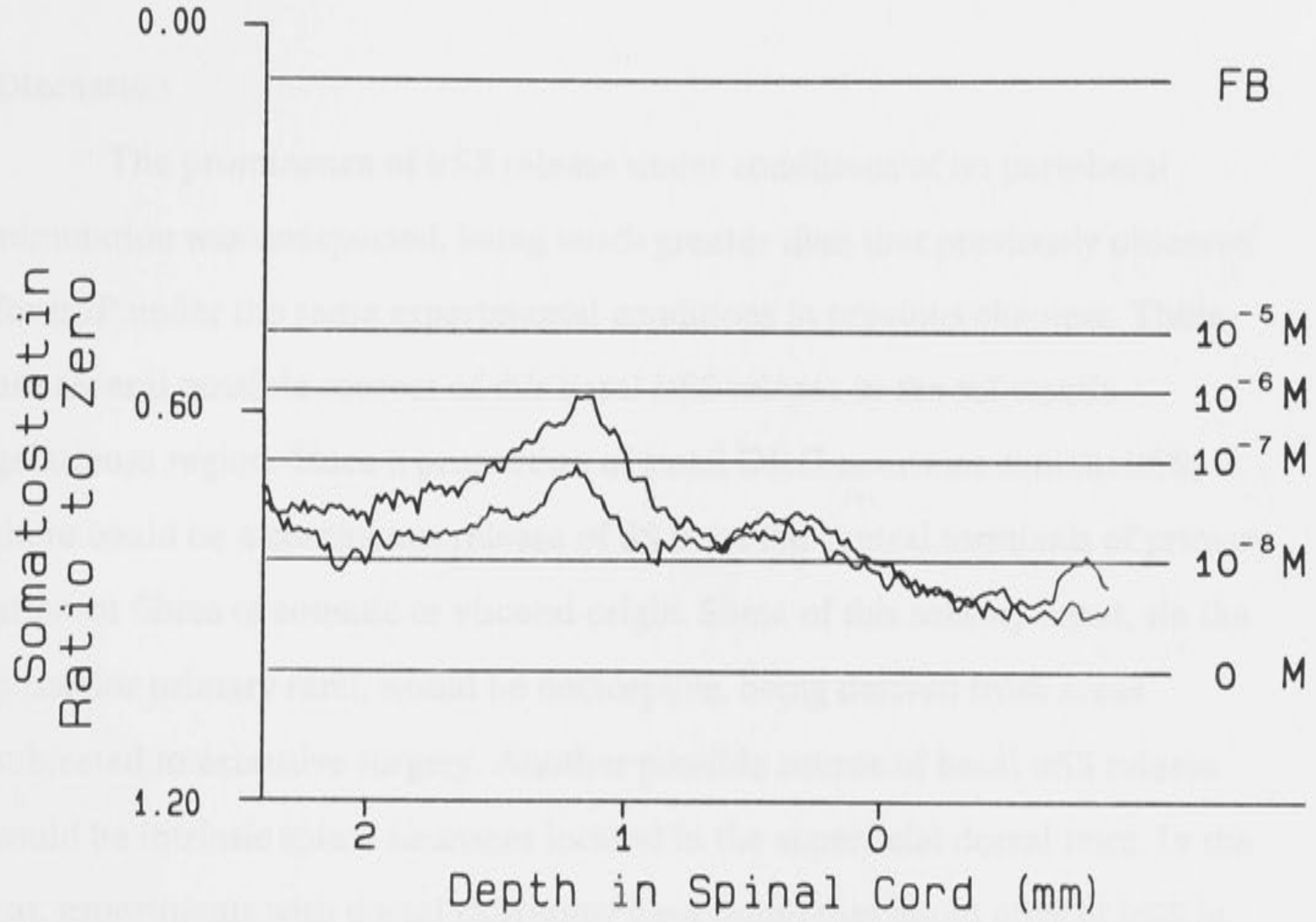


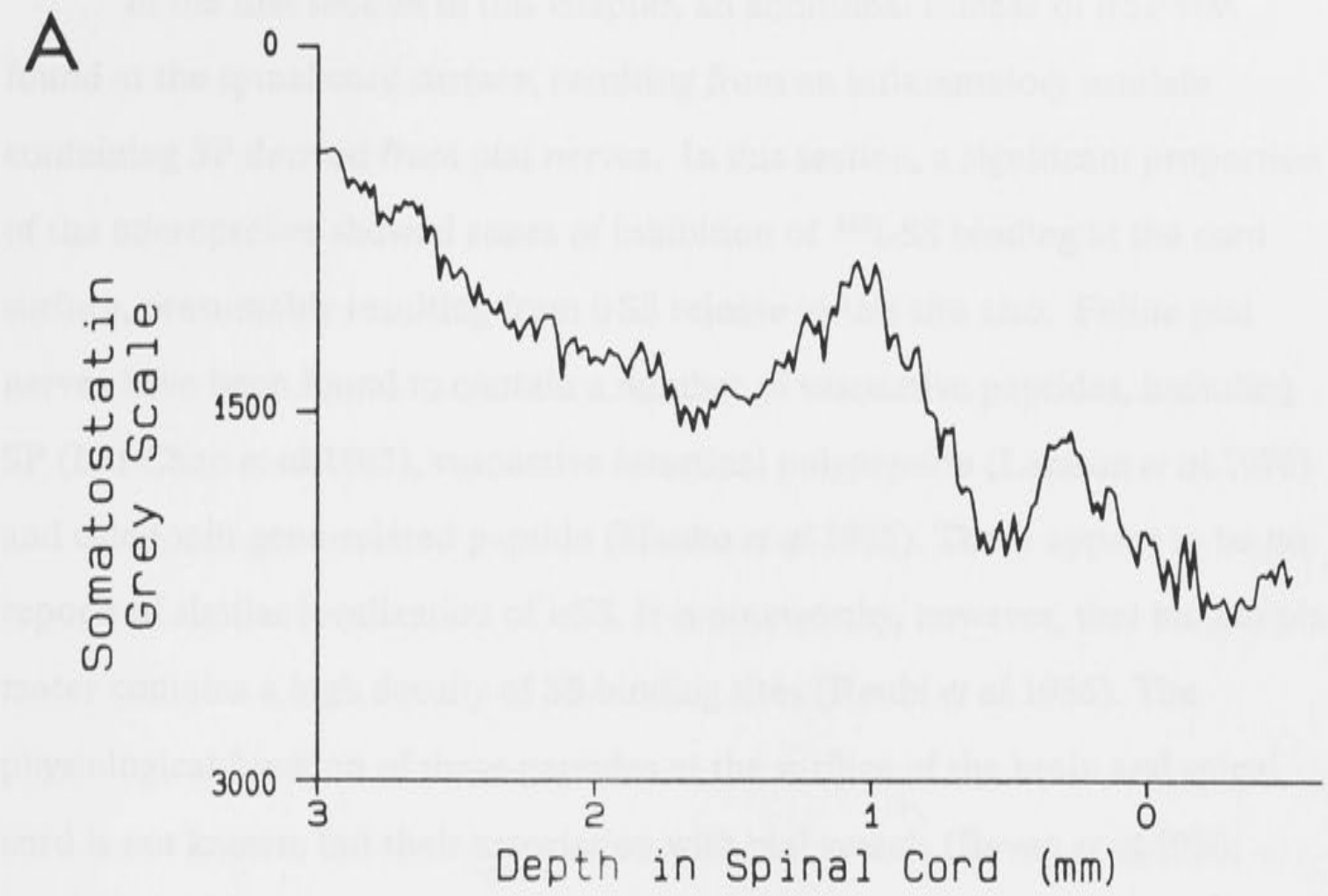
Fig. 25. Estimation of the equivalent *in vitro* concentration of irSS in the dorsal horn during cutaneous thermal stimulation. The grey scales of image density scans of microprobes exposed to standard concentrations of SS *in vitro* prior to incubation in ^{125}I-SS have been expressed as a ratio of the mean grey scale of 60 microprobes exposed only to ^{125}I-SS (*in vitro* zero group). The mean scans for each concentration were then calculated and plotted as single line averages. The image density scans of microprobes inserted in the spinal cord for 5 min during 41° C (lower scan, n = 14) or 52° C (upper scan, n = 15) stimulation of the ipsilateral hind paw have similarly been expressed as ratios to the *in vitro* zero group. Standard errors have been omitted for clarity. FB, film background.

Fig. 26 illustrates the individual image density scans of 2 such microprobes, inserted together into the lower lumbar cord during noxious heating of the hind paw (52 °C) for 5 min. Both feature a peak of release in the region of the substantia gelatinosa.

Discussion

The prominence of irSS release under conditions of no peripheral stimulation was unexpected, being much greater than that previously observed for irSP under the same experimental conditions in previous chapters. There are several possible sources of this basal irSS release in the substantia gelatinosa region. Since a proportion of small DRG neurones contain irSS, there could be a continuous release of SS from the central terminals of primary afferent fibres of somatic or visceral origin. Some of this sensory input, via the posterior primary rami, would be nociceptive, being derived from areas subjected to extensive surgery. Another possible source of basal irSS release could be intrinsic spinal neurones located in the superficial dorsal horn. In the cat, experiments with dorsal rhizotomy have found that about 60% of irSS in the dorsal horn is of intraspinal origin (Tessler *et al.* 1986), with the irSS-positive neurones located in the substantia gelatinosa region (Krukoff *et al.* 1986).

The basal release of irSS in the region of the substantia gelatinosa was not significantly altered by electrical stimulation of large myelinated afferents of the tibial nerve but was increased when C fibres were additionally activated. The SS antiserum used in the present experiments was C-terminal directed and therefore did not distinguish between SS-14 and its N-terminal-extended forms, SS-25 and SS-28. In the cat both SS-14 and SS-28 have been found in peripheral nerve, dorsal root ganglia and the upper dorsal horn (Tessler *et al.* 1986). The predominant form of SS in the cat dorsal horn, however, is SS-14 (Tessler *et al.* 1986).



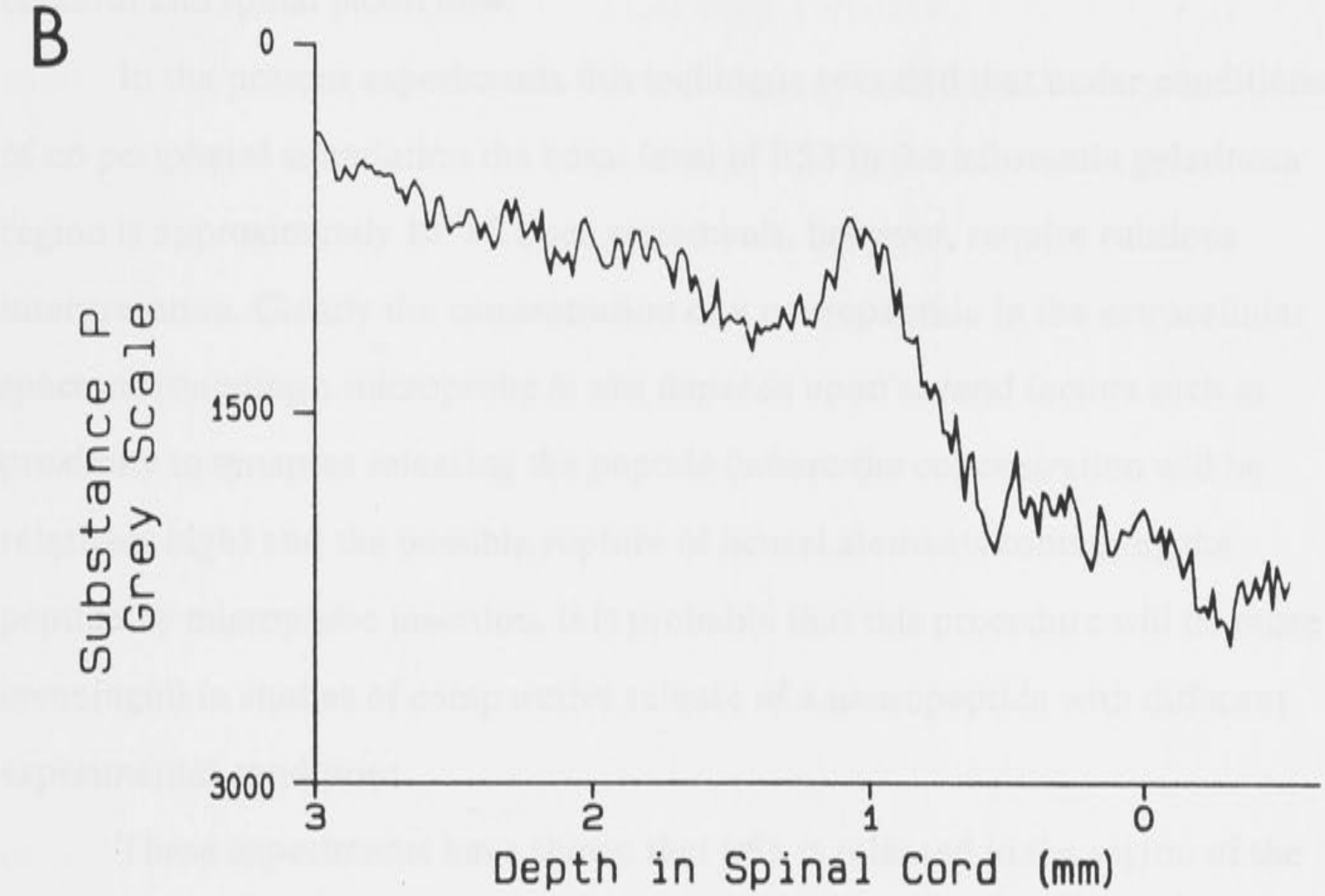


Fig. 26. Simultaneous release of irSS and irSP in the substantia gelatinosa.

A,B: Image density scans of single microprobes detecting either irSS (A) or irSP (B) release in the lumbar dorsal horn. Both microprobes were inserted concomitantly and in close proximity to each other in the spinal cord for 5 min during immersion of the ipsilateral hind paw in water at 52°C.
(With microprobe A, irSS release was also detected near the dorsum of the spinal cord).

In the first section of this chapter, an additional release of irSP was found at the spinal cord surface, resulting from an inflammatory exudate containing SP derived from pial nerves. In this section, a significant proportion of the microprobes showed zones of inhibition of ^{125}I-SS binding at the cord surface, presumably resulting from irSS release at this site also. Feline pial nerves have been found to contain a number of vasoactive peptides, including SP (Liu-Chen *et al.* 1983), vasoactive intestinal polypeptide (Larsson *et al.* 1976) and calcitonin gene-related peptide (Hanko *et al.* 1985). There appear to be no reports of similar localization of irSS. It is noteworthy, however, that human pia mater contains a high density of SS binding sites (Reubi *et al.* 1986). The physiological function of these peptides at the surface of the brain and spinal cord is not known, but their association with pial vessels (Bevan *et al.* 1986; Edvinsson *et al.* 1981; McCulloch *et al.* 1986) suggests a regulatory role in cerebral and spinal blood flow.

In the present experiments this technique revealed that under conditions of no peripheral stimulation the basal level of irSS in the substantia gelatinosa region is approximately 10^{-7}M. Such statements, however, require cautious interpretation. Clearly the concentration of a neuropeptide in the extracellular space surrounding a microprobe *in situ* depends upon several factors such as proximity to synapses releasing the peptide (where the concentration will be relatively high) and the possible rupture of neural elements containing the peptide by microprobe insertion. It is probable that this procedure will be more meaningful in studies of comparative release of a neuropeptide with different experimental conditions.

These experiments have shown that irSS is released in the region of the substantia gelatinosa of the dorsal horn in response to noxious cutaneous thermal, but not mechanical, stimulation. This is an important difference to the release, in the same spinal region, of irSP and irCGRP which is evoked by both types of noxious cutaneous stimuli (*vide infra*; Tsai *et al.* 1988). For SS the

specificity in relation to the type of noxious stimulus producing release suggests that the source of the irSS is not polymodal nociceptors but rather, specialized heat nociceptors. Electrophysiological studies have described the presence of such receptors in the skin of the cat hind paw, with the afferent fibres occurring in the plantar and saphenous nerves (Iggo, 1959; Beck *et al.* 1974). In one study, similar numbers of polymodal nociceptors and 'C-heat-nociceptors' were found in cat plantar nerve (Beck *et al.* 1974). The present results suggest that these thermal nociceptors contain and release irSS, which is consistent with previous work showing irSS release in the substantia gelatinosa following electrical stimulation of unmyelinated primary afferents of the tibial nerve.

Unfortunately, attempts to relate the peptide content of nociceptors with their physiological response characteristics have been few and the results are not readily interpreted. Leah *et al.* (1985a) described a small number of cat DRG neurones containing irSS but found little correlation with the type of cutaneous stimuli exciting these cells. Such findings are complicated not only by the co-existence of other peptides (bombesin, cholecystokinin) in these neurones but also by species differences. In the cat most of the irSS-containing cells also contain irSP but many other DRG cells stain positively for irSP but not irSS (Leah *et al.* 1985b). These observations, together with the present and previous release results, suggest that in the cat, polymodal nociceptors contain and release irSP but not irSS, while the subset of DRG neurones containing both these peptides might be the specialized heat nociceptors (Beck *et al.* 1974). The present work is consistent with an earlier report of intraspinal irSS release detected with the push-pull cannula technique (Kuraishi *et al.* 1985). Such release was evoked by noxious cutaneous thermal, but not mechanical stimulation at an unstated site within the dorsal horn. With the antibody microprobe technique, the intraspinal sites of irSS release have been localized to the substantia gelatinosa, a region where SS binding sites are concentrated (Reubi & Maurer, 1985; Reubi *et al.* 1986). The present study is also in general

accord with the results of behavioural experiments showing that intrathecal SS administration produced reactions suggestive of pain perception (Wiesenfeld-Hallin, 1985, 1986a,b) but see (Mollenholt *et al.* 1988), and potentiated a flexion reflex to noxious thermal but not mechanical information (Wiesenfeld-Hallin, 1986b). Collectively these findings suggest that SS is an excitatory transmitter of noxious thermal information. This released SS could be of primary afferent origin since, in the cat, DRG neurones contribute about 40% of the dorsal horn content of irSS (Tessler *et al.* 1986). Alternatively, irSS could equally be released form intrinsic spinal neurones of the substantia gelatinosa which terminate locally. To explain the present results, such cells would require a selective excitation by noxious thermal afferent input.

Although irSS is released during thermal nociception, this peptide has been reported to have inhibitory effects in the spinal cord. Administration of SS has inhibited dorsal horn neurones of the rat spinal cord *in vitro* (Miletic & Randic, 1982; Murase *et al.* 1982) and the cat spinal cord *in vivo* (Randic & Miletic, 1978), and intrathecal administration has produced analgesia (Chrubasik *et al.* 1984; Chrubasik *et al.* 1985). In addition to anti-nociceptive effects, however, intrathecal SS produces hind limb paralysis and neuronal damage (Long, 1988; Mollenholt *et al.* 1988). Therefore, it is possible that at least some of the reported inhibitory and analgesic effects of SS in the spinal cord result from and ischaemic neurotoxic action of this neuropeptide.

Although the main advantages of the antibody microprobe are spatial precision and lack of trauma, estimations of the equivalent *in vitro* concentration of peptide present in regions of release are a useful adjunct to the technique. Thus under conditions of no peripheral stimulation or innocuous cutaneous stimulation, the concentration of irSS detected in the substantia gelatinosa region was equivalent to an *in vitro* concentration of 10^{-7}M. With noxious heating of the skin, the equivalent concentration of irSS detected in this region was 10-fold greater, 10^{-6}M. This concentration of SS has produced

membrane potential changes in dorsal horn neurones of the rat spinal cord *in vitro* (Murase *et al.* 1982). Such estimates of concentration thus provide an additional basis for assessing the significance of a particular zone of release in response to various peripheral stimuli.

In summary, the present experiments have identified 2 regions of irSS release within the lumbar spinal cord of the cat, the substantia gelatinosa and the overlying pia mater. The increase in irSS release in the substantia gelatinosa produced by impulses evoked electrically in unmyelinated primary afferents and with thermal but not mechanical cutaneous stimulation suggests that SS plays a specialized role in spinal nociceptive processing.

Opioid Peptide Release

Results

Dynorphin A release and effect of cord transection

A basal release of ir-dynorphin A was observed in the region of lamina I. **Fig. 27** (lower scan) illustrates the mean scan of antibody microprobes detecting ir-dynorphin A in the absence of peripheral stimulation. A small peak is evident 1.0 mm from the cord dorsum which corresponds to the centre of lamina I in the cat lumbar dorsal horn. The average depth of the zone of inhibition of tracer binding was determined by obtaining a mean scan of all microprobes aligned from their tips. Because of the variation in the depth of particular laminae from the cord dorsum, the small zone of release in lamina I was highlighted by placing all zones at 1.0 mm from the cord surface. The upper scan in **Fig. 27** shows the effect of spinal transection. The lamina I peak was no longer present on the mean scan line, but a larger peak of inhibition of tracer binding was observed near the surface of the cord dorsum. Measures of the AUS in lamina I for individual scans before cord transection gave a mean value of 123.4 ± 43.3 (SEM, n = 16) GS x mm, which was significantly reduced

SUPRASPINAL CONTROL OF irDYNORPHIN A RELEASE

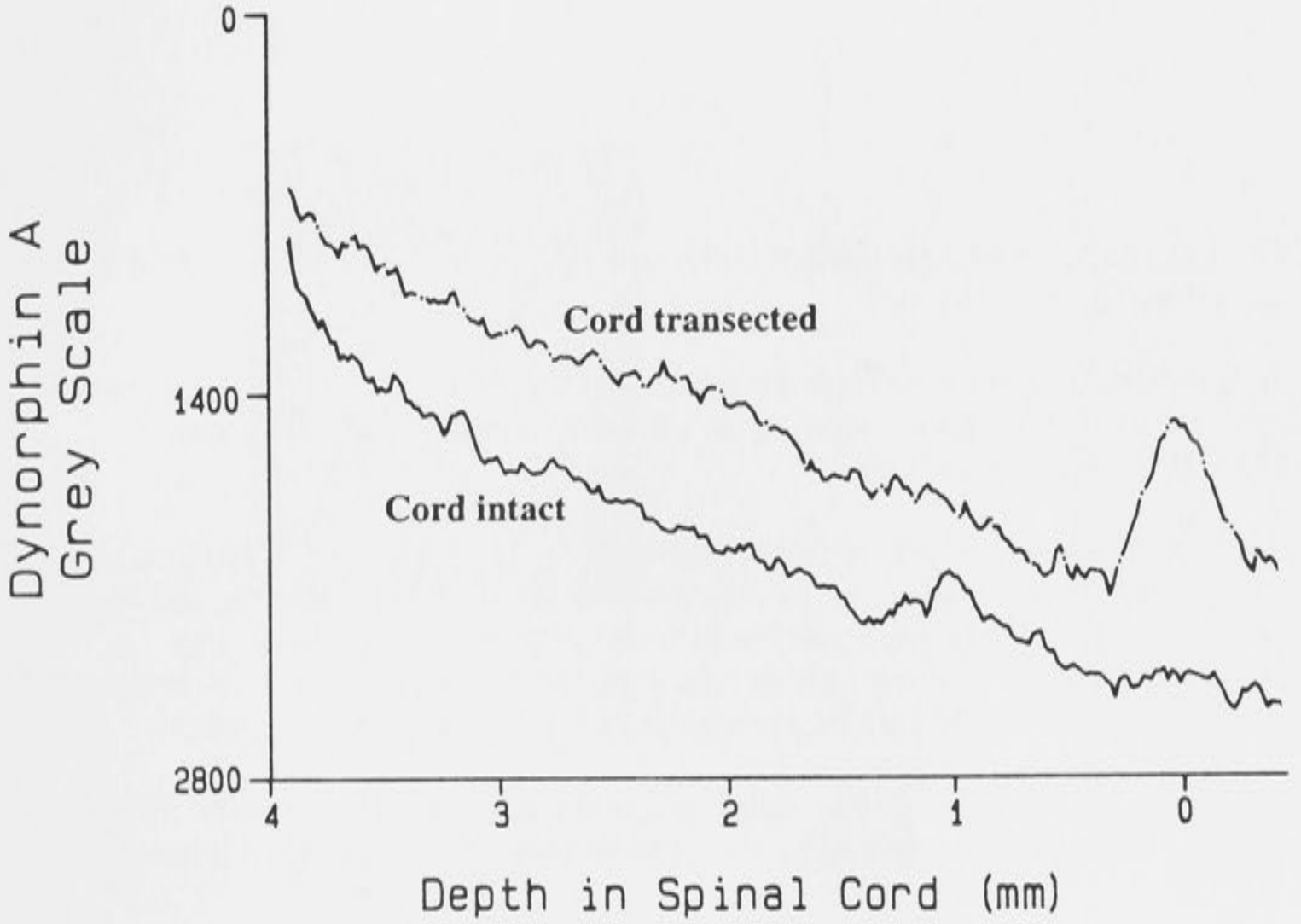


Fig. 27. Supraspinal control of ir-dynorphin A release in lamina I of the lumbar spinal cord.

Lower line: Average image density scan for 16 microprobes inserted 4.0mm into the intact cord for 30 min.

Upper line: Average image density scan for 12 microprobes treated as above but with the cord transected at the thoracolumbar level. Both results were obtained in the absence of somatic or nerve stimulation. Standard error lines have been omitted for clarity. See Results section for mean ± S.E.M. for the AUS at the level of lamina I for these two groups.

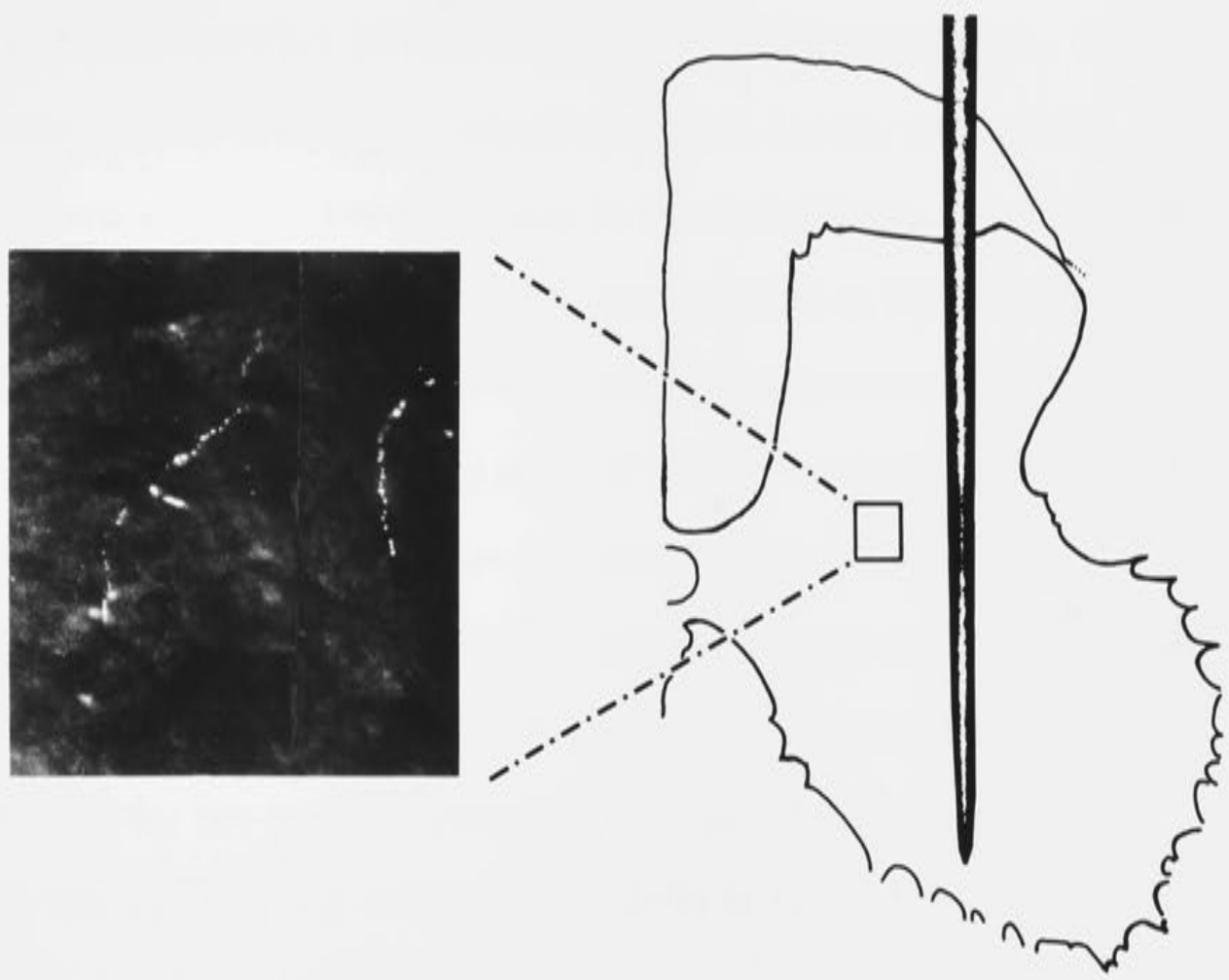
Fig. 28. Localization and release of ir-dynorphin A (1-17) in lamina VI detected with an antibody microprobe.

A: Left hand side. Immunohistochemical localization of ir-dynorphin A terminals confirms the site of release in lamina VI. Vertical height of the photo inset corresponds to a scale of 300μm.

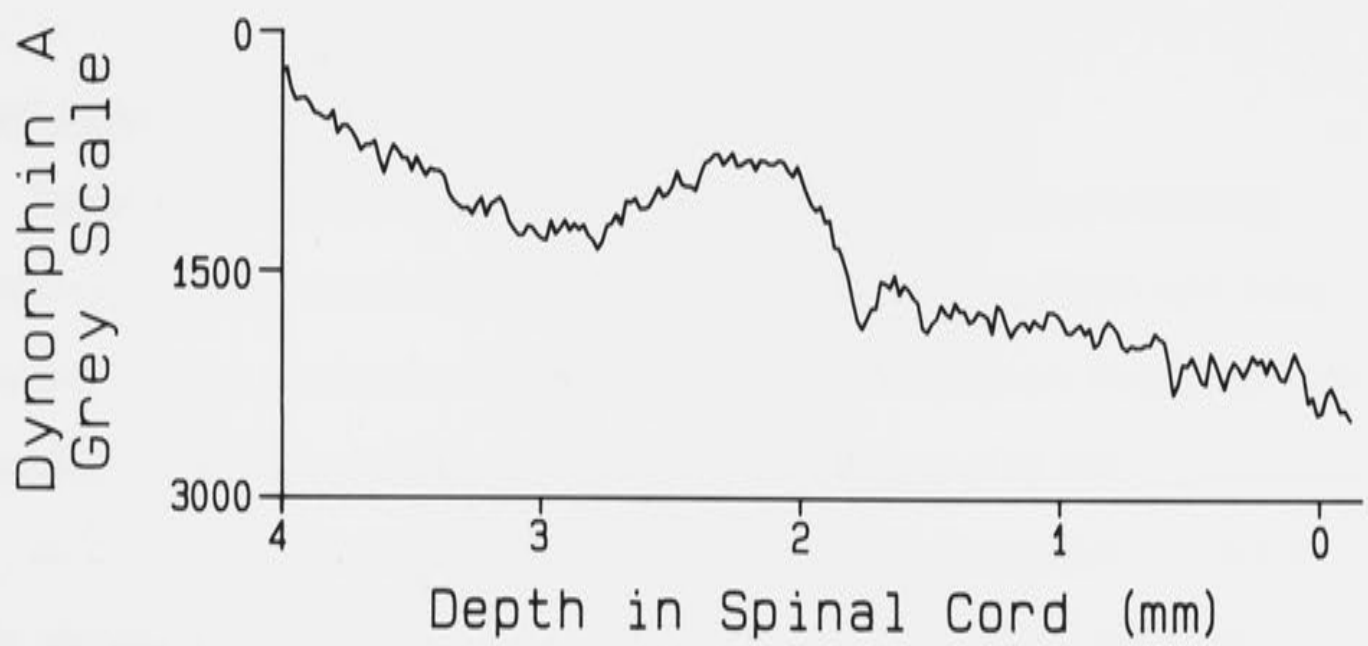
Right hand side: A photographic enlargement of the autoradiographic image of a microprobe placed on a diagram of the spinal cord. Radiolabeled dynorphin A bound to the microprobe exposed the film which shows as white on the photographic print. This microprobe was inserted 4.0 mm into the lower lumbar cord of an intact cat for 30 min in the absence of any somatic stimulation.

B: The image density scan of the microprobe shown in A. The zone of inhibition of binding of radiolabeled dynorphin A corresponds to the region of lamina VI.

A



B


Dynorphin A
Grey Scale
0
1500
3000
4
3
2
1
0
Depth in Spinal Cord (mm)

($P < 0.05$) to an AUS of 22.2 ± 7.23 (SEM, n = 12) GS x mm after cord transection.

There was some evidence for release of ir-dynorphin A in lamina VI. The right hand portion of **Fig. 28A** illustrates a photographic enlargement of the autoradiographic image of a single antibody microprobe which was inserted 4.0 mm into an intact spinal cord for 20 min with no peripheral stimulation. There is a zone of release which, on the corresponding optical density scan of this microprobe, is shown as a peak centered deep in the dorsal horn, about 2.2 mm from the dorsal cord surface (**Fig. 28B**). This corresponds to Rexed's lamina VI where, as shown in the inset of **Fig. 28A** , dynorphin A immunoreactive terminals were subsequently found. This zone of release was very pronounced in one experiment (**Fig. 29A**), but was not consistently observed in later experiments. However, as with ir-dynorphin A release in lamina I (**Fig. 27**), the zone of release in lamina VI was absent after the cord was transected (**Fig. 29B**). Thus, the AUS for the peak in lamina VI with an intact spinal cord was 240 ± 47.9 which was significantly reduced ($P < 0.01$) to an AUS of 55.8 ± 17.5 in the spinal cat.

Evoked release of dynorphin A

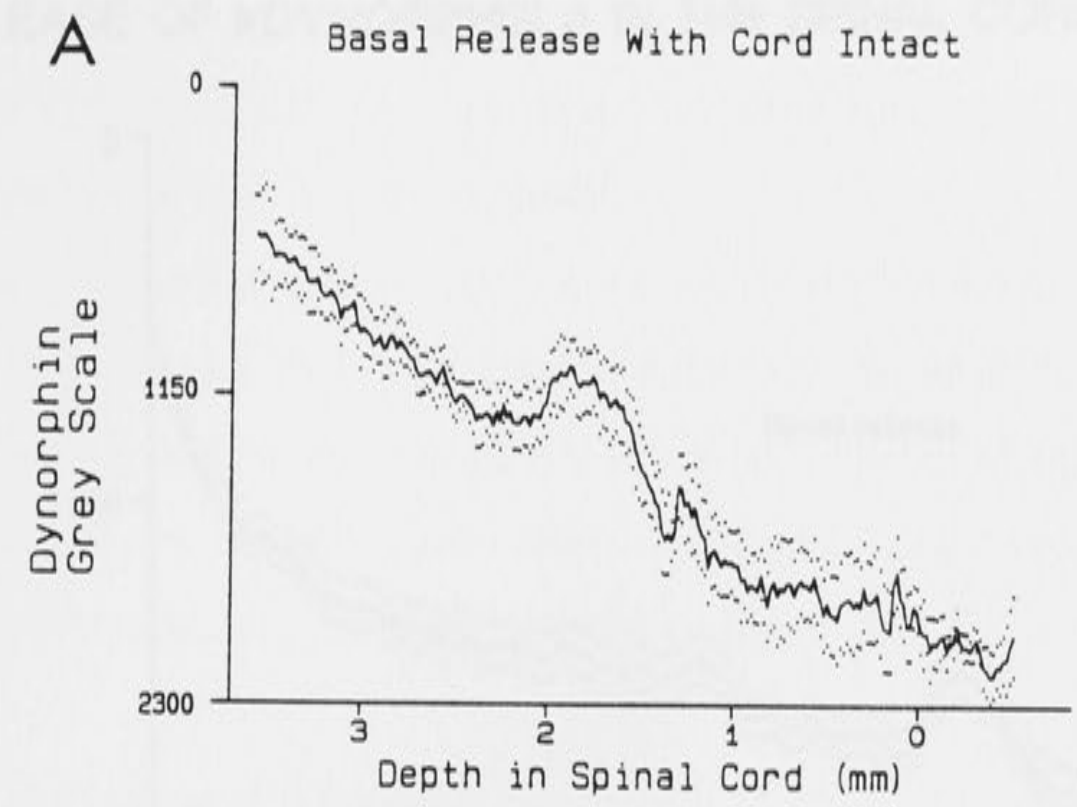
In view of the published reports of dynorphin A release following electroacupuncture stimulation (Han & Xie, 1984; Fei *et al.* 1986) and work suggesting that the analgesic effects of electroacupuncture are mediated in part by impulses in Aδ afferent fibres (Woolf *et al.* 1980; Lee *et al.* 1985; Sjölund, 1985), antibody microprobes were used to measure ir-dynorphin A release during stimulation of ipsilateral peripheral nerves. The spinal cord was not transected in these experiments. **Fig. 30** shows that there was no effect of stimulation of the sural nerve at intensities sufficient to excite Aδ afferent fibres. Both scans have small peaks in the area of lamina I and much larger peaks near the surface of the cord. The broad peak seen on the mean scan at the cord

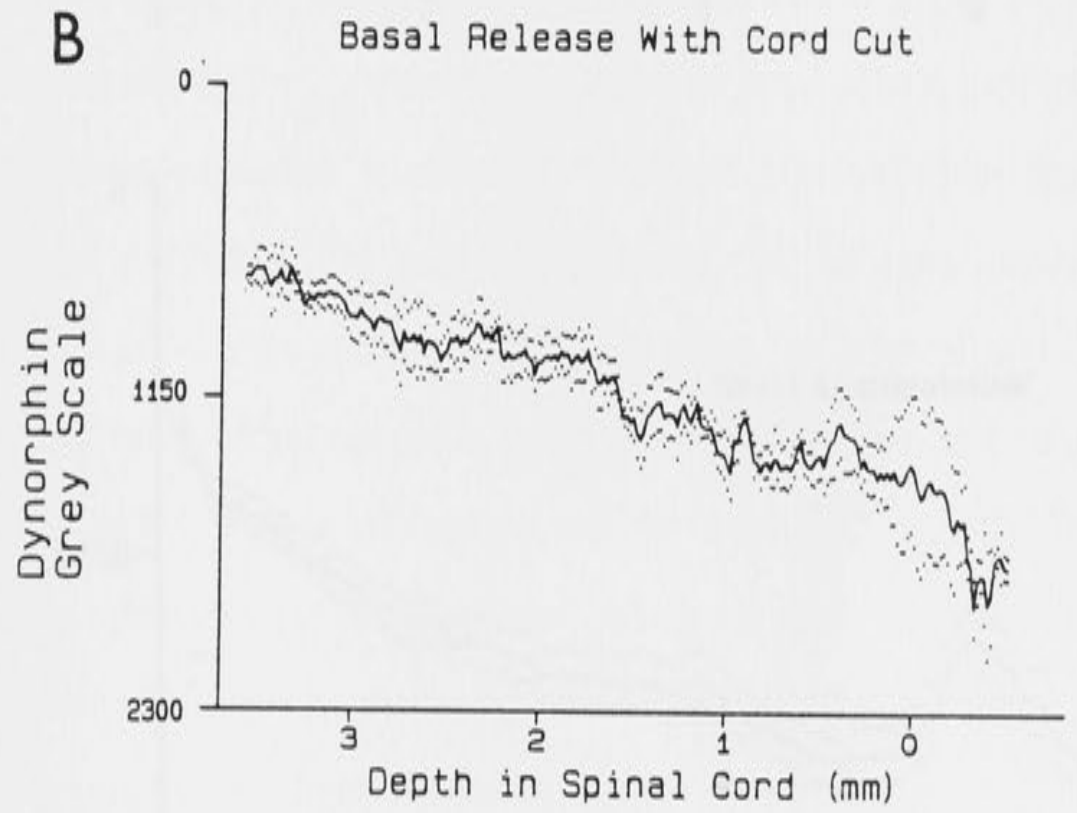
Fig. 29. Supraspinal control of ir-dynorphin A release in lamina VI and lack of C afferent fibre stimulation to evoke ir-dynorphin A release in the dorsal horn.

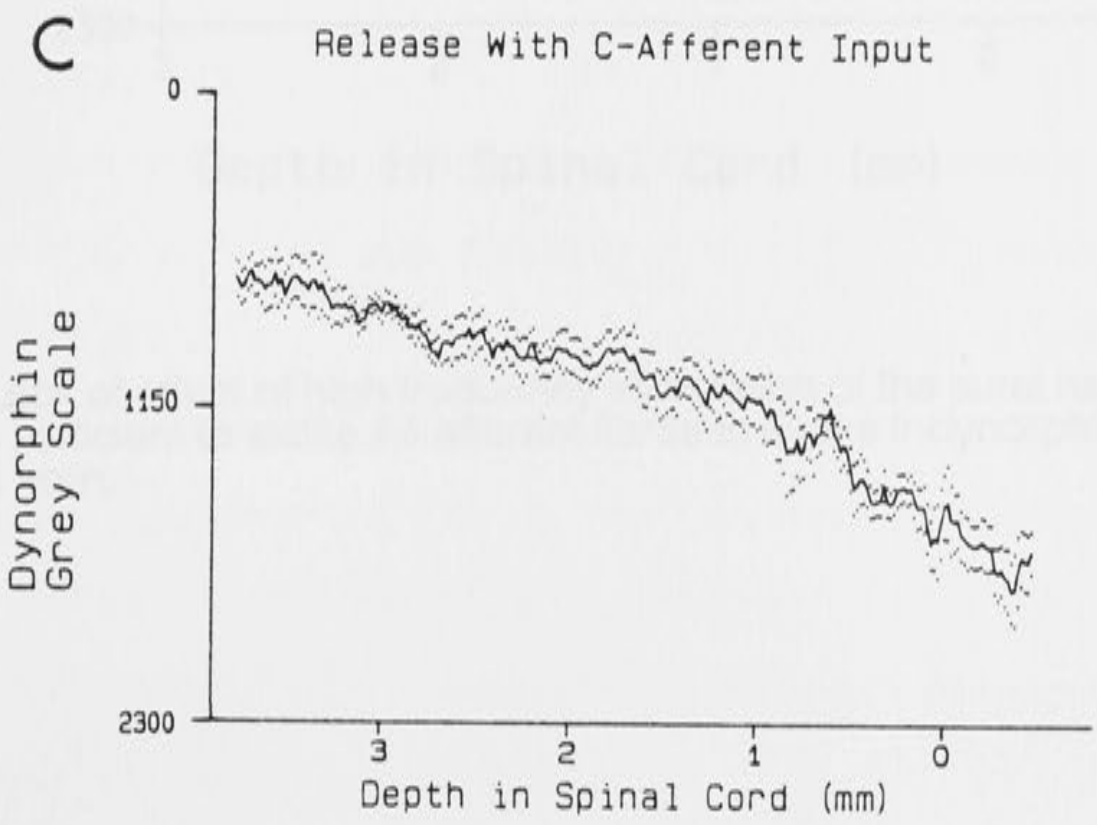
A: The mean image scan (±S.E.M.) of 6 microprobes inserted 4.0mm into the intact spinal cord for 30 or 40 min in the absence of nerve stimulation.

B: The mean image scan (±S.E.M.) of 7 microprobes inserted 4.0mm into the spinal cord for 30 or 40 min during and after cord transection. The test of significance for the difference **A - B** and is not shown.

C: The mean image scan (±S.E.M.) of 12 microprobes inserted 4.0mm into the spinal cord for 30 or 40 min during 100 Hz continuous stimulation (0.5 ms pulse width) of the tibial nerve at intensities sufficient to excite C fibres after cord transection.


A
Basal Release With Cord Intact
0
1150
2300
Dynorphin
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)


B
Basal Release With Cord Cut
0
1150
2300
Dynorphin
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)


C
Release With C-Afferent Input
0
1150
2300
Dynorphin
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)

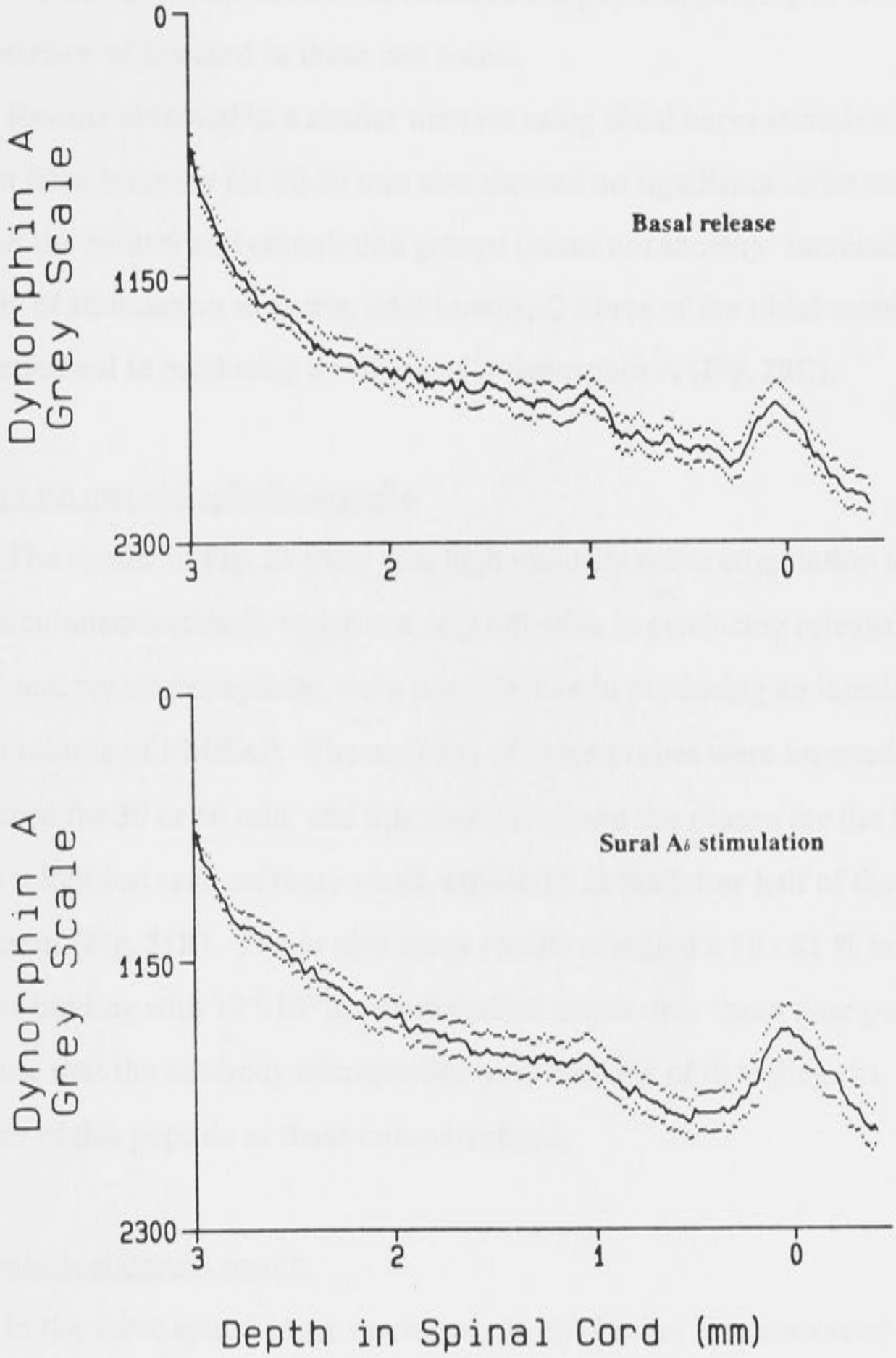


Fig. 30. Lack of effect of high frequency stimulation of the sural nerve at intensities sufficient to excite Aδ afferent fibres to evoke ir-dynorphin A release in the dorsal horn.

surface is due to the averaging of quite narrow peaks seen on individual scans. There was no significant difference between the peaks appearing in lamina I or at the surface of the cord in these two scans.

Results obtained in a similar manner using tibial nerve stimulation at Aδ afferent fibre intensity for 20-30 min also showed no significant differences between the control and stimulation groups (scans not shown). Increasing the intensity of stimulation to excite, additionally, C fibres of the tibial nerve was also ineffectual in producing a release of ir-dynorphin A (**Fig. 29C**).

Studies with met-enkephalin-arg-phe

The results in **Fig. 31** show that high intensity nerve stimulation and noxious cutaneous stimuli, which are very effective in producing release of several sensory neuropeptides, were not effective in producing an intralaminar zone of release of irMEAP. The majority of these probes were inserted in the spinal cord for 30 or 60 min, and this may have been the reason for the large surface inhibition seen on these scans, especially in the latter half of the experiment (**Fig. 31B**). The *in vitro* assay results revealed a 50 - 81 % inhibition of tracer binding with 10^{-7}-10^{-5}M of unlabelled tracer over these time periods, validating that the antibody microprobes were capable of detecting the presence of this peptide at these concentrations.

Immunohistochemical results

In the three spinal cords examined, no cell bodies immunoreactive for dynorphin A were found and this is probably due to the lack of colchicine pretreatment which was deemed incompatible with release experiments. Dynorphin A immunoreactive terminals were occasionally encountered in these studies in transverse sections of the spinal cord. This immunoreactivity was localized in boutons in lamina VI (**Fig. 28A**), and a few terminals were seen in more superficial laminae. Sections stained for irMEAP showed quite different

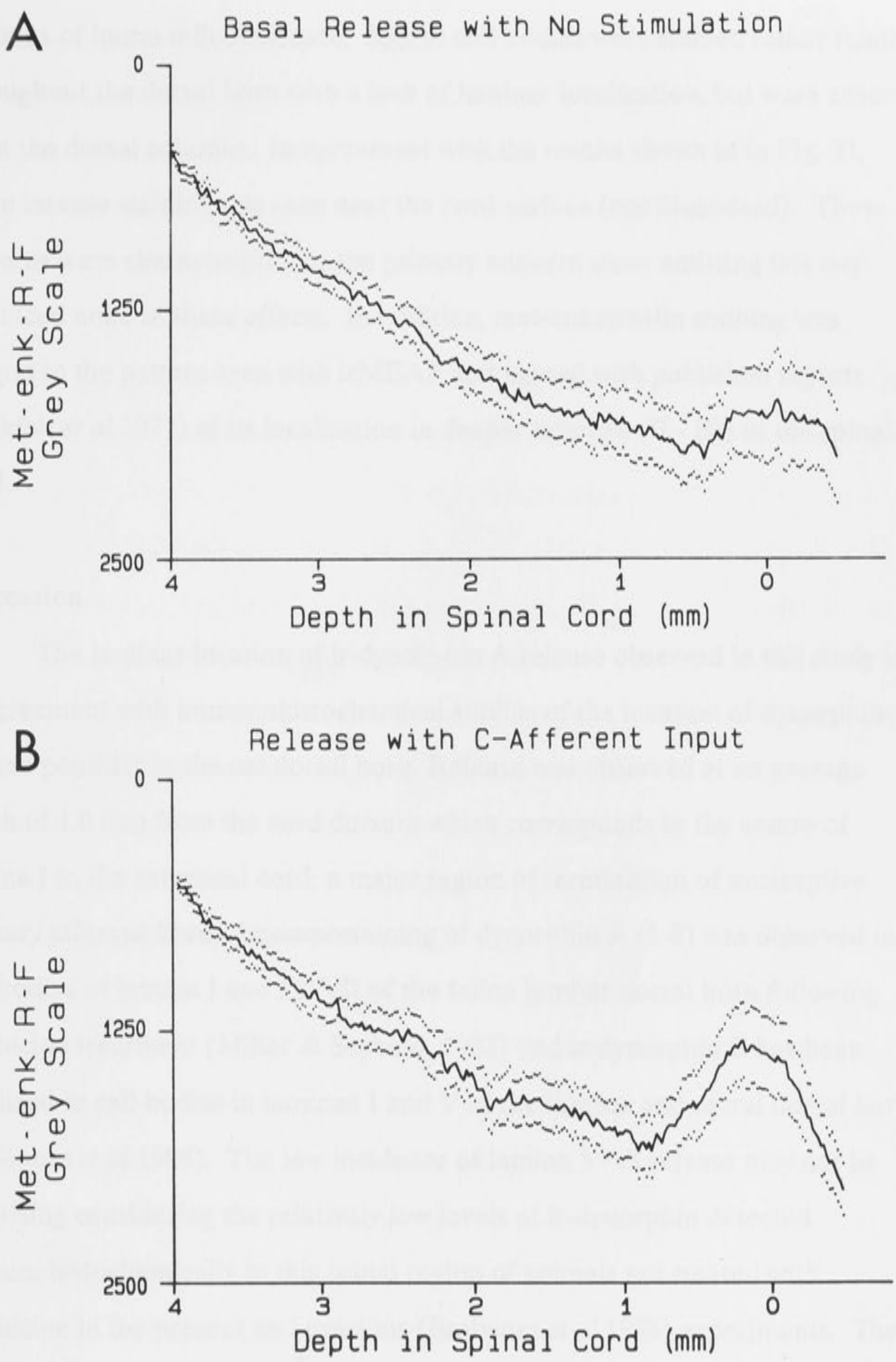


Fig. 31. Met-enkephalin-arg-phe microprobes show prominent surface inhibition but no intralaminar release with noxious afferent input.

A: The mean image scan (±S.E.M.) of 17 microprobes inserted 4.0mm into the spinal cord for 30 or 60 min in the absence of stimulation.

B: The mean image scan (±S.E.M.) of 20 microprobes inserted 4.0mm into the spinal cord. Pooled results of various stimuli: 10 Hz tibial nerve stimulation at C fibre intensity (N = 12); noxious mechanical (N = 4); 52^{o}C hot water (N = 4) for 30 or 60 min.

patterns of immunofluorescence. Sparse cell bodies were stained rather faintly throughout the dorsal horn with a lack of laminar localization, but were absent from the dorsal columns. In agreement with the results shown in in **Fig. 31**, more intense staining was seen near the cord surface (not illustrated). These patterns were characteristic for the primary antisera since omitting this step produced none of these effects. In addition, met-enkephalin staining was unique to the pattern seen with irMEAP and agreed with published reports (Hökfelt *et al.* 1977) of its localisation in deeper laminae (II - III) of the spinal cord.

Discussion

The laminar location of ir-dynorphin A release observed in this study is in agreement with immunohistochemical studies of the location of dynorphin-related peptides in the cat dorsal horn. Release was observed at an average depth of 1.0 mm from the cord dorsum which corresponds to the centre of lamina I in the cat spinal cord; a major region of termination of nociceptive primary afferent fibres. Immunostaining of dynorphin A (1-8) was observed in cell bodies of lamina I and IV-VII of the feline lumbar dorsal horn following colchicine treatment (Miller & Seybold, 1987) and ir-dynorphin B has been localized in cell bodies in laminae I and V of the lumbar and sacral dorsal horns (Basbaum *et al.* 1986). The low incidence of lamina V-VI release may not be surprising considering the relatively low levels of ir-dynorphin detected immunohistochemically in this spinal region of animals not treated with colchicine in the present and previous (Basbaum *et al.* 1986) experiments. The induction of arthritis or other forms of chronic stress increases dynorphin mRNA levels (Höllt *et al.* 1987; Ruda *et al.* 1988) and more consistent release from lamina V-VI may be obtained under these conditions. A recent immunohistochemical study has identified ir-calcitonin-gene-related peptide in varicosities presumed to derive from primary afferent fibres which contact

dendrites and cell bodies of ir-dynorphin A (1-8) neurones in laminae I, II and V-VI (Takahashi & Traub, 1988). The incidence of these synaptic contacts was markedly increased in the arthritic rat (Takahashi & Traub, 1988). This supports the notion that dynorphin-containing interneurones or projection neurones are involved in processing nociceptive information (Miller & Seybold, 1987).

In the present study, the observation that dynorphin A release in lamina I was abolished when the spinal cord was transected implies that supraspinal sites may be involved in the control of dynorphin release at the spinal level. Although bulbospinal projections of peptidergic neurones containing SP and enkephalin have been described in the rat, no spinally projecting ir-dynorphin B neurones were found (Menetrey & Basbaum, 1987). It is likely that release observed at lumbar levels is derived from dynorphin-containing interneurones under the influence of other descending pathways. Indeed, there is some evidence that descending noradrenergic or serotonergic fibres may control dynorphin release from spinal interneurones. Thus the addition of 10^{-4}M clonidine, an $alpha_2$ adrenoceptor agonist, to the artificial CSF perfusing the subarachnoid space of the rat stimulates the release of dynorphin A into spinal perfusates (Xie et al.1986). A serotonergic component may be involved since the prolonged response latency induced by a selective kappa receptor agonist in hot plate and tail flick assays in the rat was blocked by p-chlorophenylalanine pretreatment (Von Voigtlander et al.1984). Further support for involvement of dynorphin A in descending inhibition comes from microelectrophoretic experiments. When administered in the vicinity of feline multireceptive spinocervical tract neurons, dynorphin A(1-13) selectively reduced nociceptive responses in 27 of 31 cells tested (Fleetwood-Walker et al.1988). Such selective effects are characteristic of descending inhibition present on these spinal neurones in this anaesthetized cat preparation (Duggan & Morton, 1988).

Spinal cord levels of dynorphin have been found to be elevated 1-4 days after spinal trauma (Cox *et al.* 1985; Faden *et al.* 1985b) but unaltered 12-32 days following cord transection (Goldstein & Ghazarossian, 1980). These results are not necessarily discordant with the present observations following acute cord transection since an early phase of cessation of release may be followed initially by a period of increased synthesis, and a subsequent return to normal levels. The determination of dynorphin levels is a static measure at one point in time, and it is not possible to judge the relative contribution of synthesis, release and degradation to the observed content.

An important finding of the present work was absence of enhanced ir-dynorphin A release in the lumbar cord following electrical stimulation of peripheral nerves at intensities sufficient to excite Aδ primary afferent fibres. This is in contrast to the work of Han's group (Han & Xie, 1984), where transcutaneous nerve stimulation at similar intensities produced an analgesic effect blocked by intrathecal administration of anti-dynorphin antibody in rabbits, and released ir-dynorphin A into the spinal perfusates of rats (Fei et al.1986). Although it is uncertain to what extent dynorphin A produces the analgesic effects of transcutaneous nerve stimulation, the present results indicate that there may be species differences with regard to the intraspinal release of this peptide under conditions of analgesia induced by peripheral nerve stimulation.

The origin of release at the cord surface is at present unknown, but it appears to depend on the time of insertion rather than on a particular stimulus (see discussion on pp. 42-43). In **Fig. 27**, this zone of release appears with the group of probes inserted after cord transection and these probes were, of necessity, all inserted about 6 h later than the control group. It is likely that both image scans in **Fig. 30** show surface release because control and stimulation microprobes were alternated over the time course of the experiment, and only the late probes in each group contributed to the peak.

However, the large surface inhibition obtained with irMEAP release may, in part, be due to authentic release of peptide since immunohistochemical fluorescence was observed in spinal cord sections corresponding to the region of release on the microprobes. This time-related appearance of surface release has been observed in previous microprobe experiments with SP. The lack of release observed in deeper lamina with irMEAP may simply be due to the low concentration and lack of laminar organization of these neurones. It seems that one requirement of the antibody microprobe technique is that the neuropeptide be localised to a discrete region. If this is the case, then one clear advantage of the technique is the ability to detect release in discrete laminae deep within the spinal cord that may not be measured by the technique of spinal superfusion.

In summary, this is the first *in vivo* study to localise ir-dynorphin A release at the laminar level within the spinal cord, and the results suggest that dynorphin A release in laminae I and VI is under a supraspinal influence. In contrast, there is no evidence that in the cat, ir-dynorphin A is released by electrical stimulation of peripheral nerves using parameters which may produce the phenomenon of electroacupuncture (Han & Terenius, 1982).

5. PHYSIOLOGICAL CONTROL OF SUBSTANCE P RELEASE

Absence of Tonic Descending Inhibition

Introduction

Presynaptic inhibition of transmission of impulses in nociceptive primary afferents has been proposed as important in several mechanisms of analgesia. These include the gate hypothesis of Melzack & Wall (1965), the actions of opiates and opioid peptides in the substantia gelatinosa (Jessell & Iversen, 1977) and the effects in the dorsal horn of descending fibres derived from brainstem nuclei (Fields & Basbaum, 1978) (see **Fig. 50** for diagram). There are some neurophysiological studies supporting these hypotheses (Hentall & Fields, 1979; Carstens et al.1979; Fitzgerald & Woolf, 1981; Calvillo et al.1982). Several anatomical reports however, argue against presynaptic inhibition as an important control of spinal transmission of impulses in peripheral nociceptors (Duncan & Morales, 1978; Ruda & Gobel, 1980; Hunt et al.1980; Glazer & Basbaum, 1981; Zhu et al.1981).

The function of presynaptic inhibition is to reduce the probability of synaptic transmission by decreasing the amount of transmitter released by an incoming impulse (Schmidt, 1971). Measures of transmitter release can thus be direct measures of the extent of such inhibition. Although the transmitter or transmitters released by any class of primary afferent fibre are still not known with certainty, evidence has been presented that SP is released in the dorsal horn in a stimulus-dependent manner following excitation of peripheral nociceptors. The finding that SP release is stimulus-dependent, suggests that this release should be reduced by presynaptic inhibition if such a process were operating on the central terminals of the relevant nociceptors.

An important inhibition which needs to be examined for a possible presynaptic control on nociceptors is the tonic inhibition of nociceptive transmission present both in decerebrate and anaesthetized cats (Wall, 1967;

Brown, 1971; Handwerker *et al.* 1975; Duggan *et al.* 1977b). In anaesthetized cats this inhibition is derived mainly from neurones of the ventrolateral medulla (Hall *et al.* 1982; Foong & Duggan, 1986) and importantly, bilateral inactivation of this area reduces not only tonic inhibition, but also the inhibition of spinal nociceptive transmission produced by electrical stimulation in the periaqueductal grey matter of the cat (Morton *et al.* 1984). Recordings from conscious cats suggest that tonic inhibition is also present in these animals (Collins, 1984). Although probably a heterogeneous process, one function of this tonic inhibition may be to reduce flexor withdrawal reflexes both to nociceptive and certain non nociceptive (Lundberg, 1982) primary afferents as a necessary prerequisite of voluntary motor performance. The present experiments have used the antibody microprobe technique to examine whether noxious stimulus-evoked release of irSP in the substantia gelatinosa of the spinal cord is subject to tonic supraspinal control.

Results

In this section, irSP release with normal spinal conduction has been compared with irSP release with conduction blocked by cooling the first lumbar segment, and irSP release was measured before and after transversely sectioning the spinal cord at the thoraco-lumbar junction. The total number of microprobes was 116.

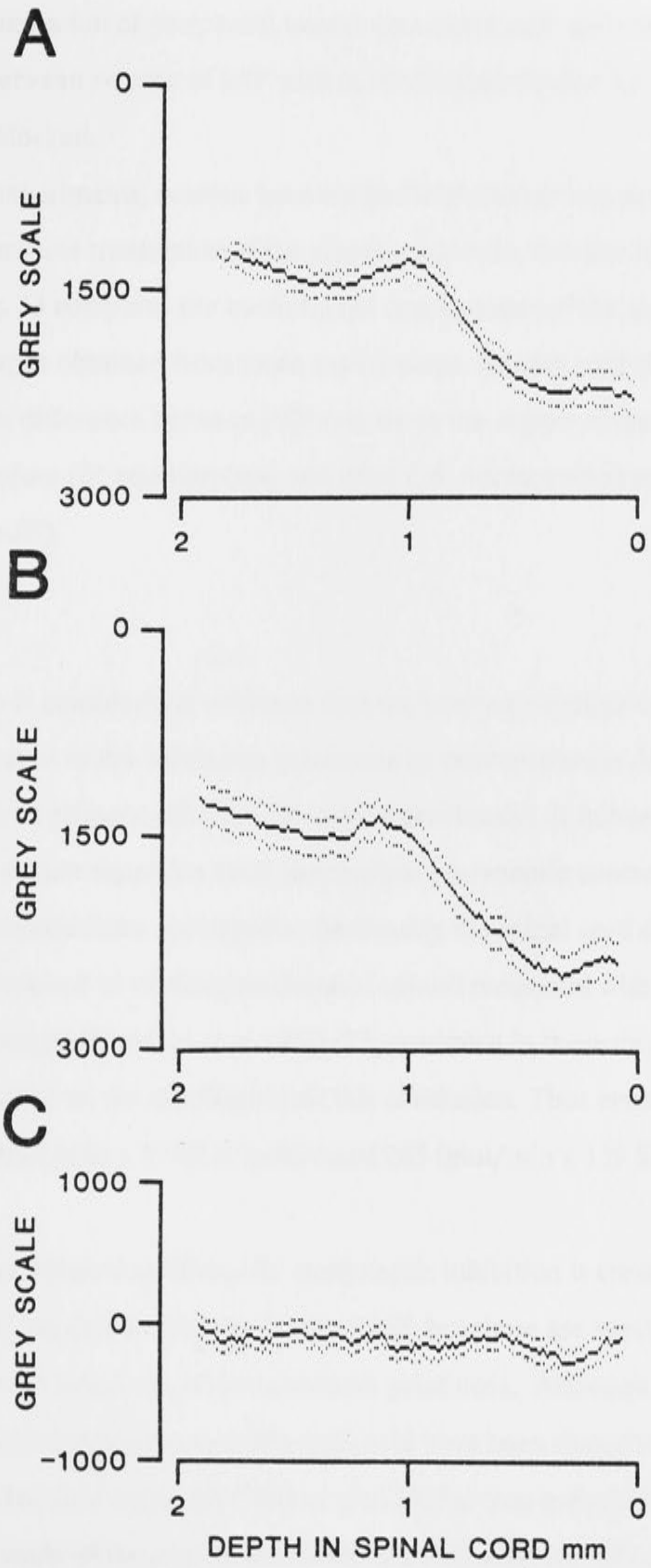
Fig. 32A illustrates the mean image density scan of the 64 microprobe autoradiographs obtained with normal spinal conduction and **Fig. 32B** shows the mean scan for the 36 microprobes used with spinal conduction blocked. Subtracting A from B gave the difference record of **Fig. 32C** . This shows that the mean image scans of microprobes with or without block of spinal conduction to be virtually identical and none of the observed differences are significant ($P > .05$). The collected data have been further analysed by considering each of the three types of noxious stimuli (thermal, mechanical and

Fig. 32. Lack of tonic supraspinal control of irSP release in the substantia gelatinosa of the spinal cord.
The illustrated means are derived from microprobes inserted 2 to 3 mm and for noxious thermal or mechanical stimuli or electrical stimulation of unmyelinated primary afferents for periods of 15 to 30 minutes.

A. The mean image scan of 64 microprobes with normal spinal conduction.

B. The mean image scan of 36 microprobes with spinal conduction blocked at the first lumbar segment.

C. The differences between scans A and B.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-1000
GREY SCALE
2
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DEPTH IN SPINAL CORD mm

electrical stimulation of peripheral nerve) separately, and again there was no difference between release of irSP with normal spinal conduction and spinal conduction blocked.

In 2 experiments, noxious heat-evoked irSP release was examined before and after complete transection of the spinal cord at the thoraco-lumbar junction. **Fig. 33** compares the mean image density scans of the microprobe autoradiographs obtained from these experiments. As with cold block there was no significant difference between irSP release in the region of the substantia gelatinosa before (21 microprobes) and after (16 microprobes) transection of the cord ($P > .05$).

Discussion

There is considerable evidence that the noxious stimulus-evoked release of irSP measured in the substantia gelatinosa by microprobes is derived from the terminals of primary afferents (discussed previously), it follows that these experiments do not support a tonic supraspinal presynaptic control of transmitter release from nociceptors. Sectioning the spinal cord did increase irSP release evoked by noxious mechanical stimuli measured with the push-pull cannula technique (Kuraishi *et al.* 1985). The variance in these data however casts some doubt on the significance of this conclusion. Thus evoked release of SP was 136 fmol/min ± 37 SEM before and 265 fmol/min ± 139 SEM after cord transection.

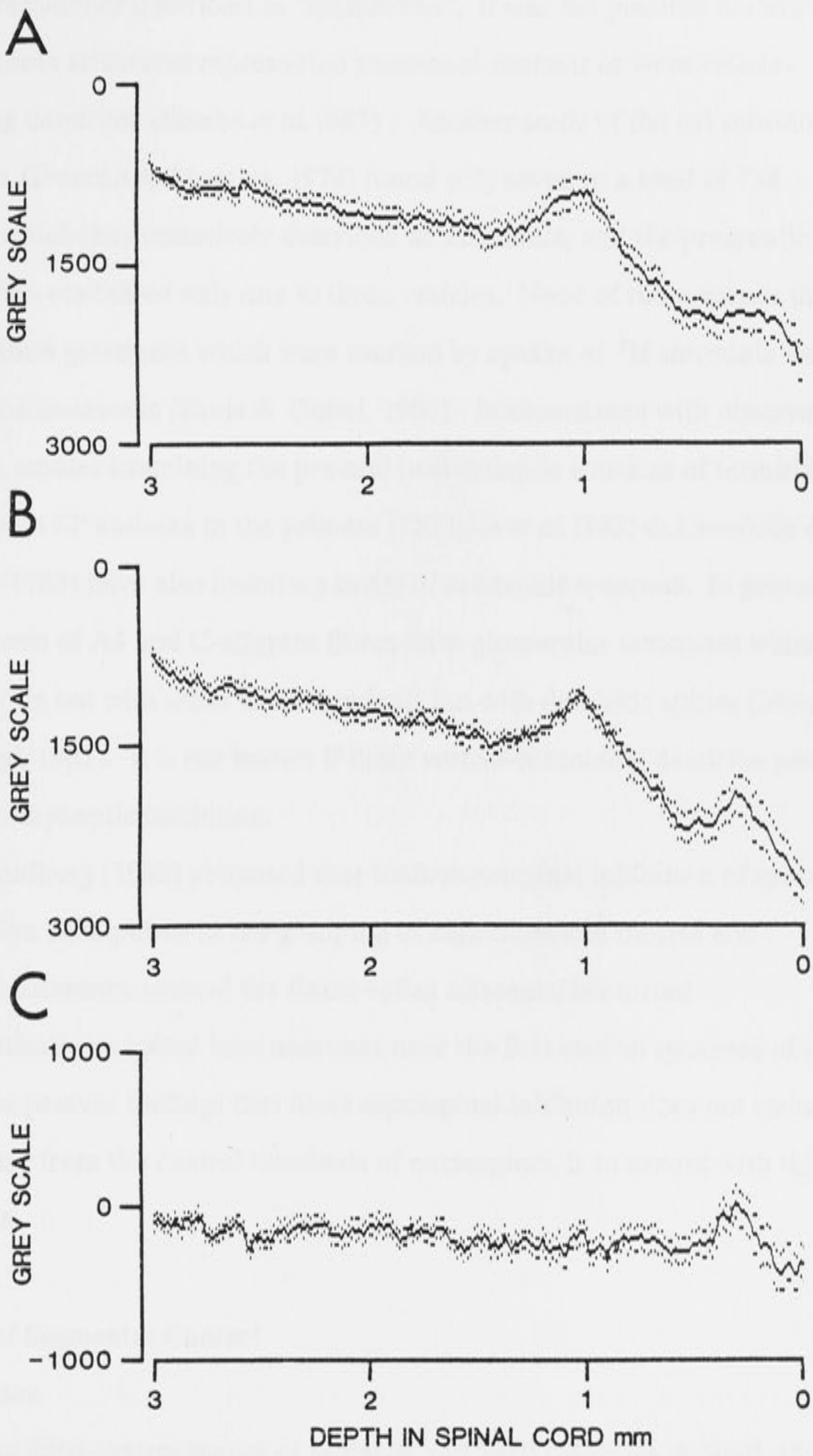
The anatomical substrate for presynaptic inhibition is considered to be Gray's type II axoaxonic synapses (Gray, 1962) but these are rarely observed on primary afferent terminals in the substantia gelatinosa. Although axoaxonic synapses containing gamma-aminobutyric acid have been described in the substantia gelatinosa of the rat (Barber *et al.* 1978a) they are relatively few in number. A study of the central terminals of slowly adapting fibres in the cat found 72% of the post-synaptic contacts were with dendritic shafts and spines

Fig. 33. Failure of spinal cord transection to alter irSP release in the substantia gelatinosa of the spinal cord.
The mean scans are derived from microprobes inserted 3 mm into the spinal cord during noxious thermal stimulation (52^{o}C) of the ipsilateral hind paw for 20 minutes.

A. The mean image scan of 21 microprobes with normal spinal conduction.

B. The mean image scan of 16 microprobes with the spinal cord transected at the thoraco-lumbar junction.

C. The differences between scans A and B.


A
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DEPTH IN SPINAL CORD mm

with the remainder described as "synapselike". It was not possible to determine whether these structures represented axoaxonal contacts or were vesicle-containing dendrites (Semba *et al.* 1983) . Another study of the cat substantia gelatinosa (Duncan & Morales, 1978) found only seven of a total of 738 synapses which they tentatively described as axoaxonic, and the presynatic profile often contained only one to three vesicles. None of the synapses in the cat substantia gelatinosa which were marked by uptake of ^{3}H serotonin were found to be axoaxonic (Ruda & Gobel, 1980). In accordance with observations in the cat, studies examining the pre-and post-synaptic contents of terminals labelled with SP antisera in the primate (DiFiglia *et al.* 1982; deLanerolle & LaMotte, 1983) have also found a paucity of axoaxonic synapses. In general, the terminals of Aδ and C-afferent fibres form glomerular structures which are post-synaptic not with other axon terminals but with dendritic spines (Maxwell & Rethelyi, 1987). It is not known if these vesicle-containing dendritic profiles mediate presynaptic inhibition.

Lundberg (1982) proposed that tonic supraspinal inhibition of spinal transmission of impulses in the grouping of high threshold muscle and cutaneous afferents, termed the flexor reflex afferents, is exerted postsynaptically on spinal interneurones near the first central synapses of these fibres. The present findings that tonic supraspinal inhibition does not reduce irSP release from the central terminals of nociceptors, is in accord with this hypothesis.

Absence of Segmental Control

Introduction

The gate-control theory of Melzack and Wall (Melzack & Wall, 1965) proposed that impulses in large diameter primary afferent fibres were capable of presynaptic inhibition of the release of transmitter from small diameter fibres. In a subsequent modification of the theory (Wall, 1978) it was stated that

existing evidence supported a presynaptic mechanism but that post-synaptic events were also likely to be involved. Several authors have concluded that both pre- and post-synaptic inhibition of dorsal horn neurones can be produced by electrical stimulation of peripheral nerves (see Besson & Chaouch, 1987) , but the relative contribution of each mechanism is a matter of controversy (see **Fig. 50**).

The previous section of this thesis has shown that irSP release is not under presynaptic control of descending fibres from supraspinal structures. In the present section, one aspect of the gate-control theory has been examined, i.e. whether activity in large diameter afferent fibres can cause a segmental presynaptic inhibition of irSP release in the dorsal horn of the cat spinal cord.

Results

In this report, a total of 121 antibody microprobes were used for calculating mean scans for various groups. In the first series of experiments, the tibial or sural nerve was stimulated at intensities sufficient to excite only A$\alpha\beta$ afferent fibres during noxious cutaneous stimulation of the ipsilateral hind paw. In a second series of experiments, the intensity of nerve stimulation was increased so as to excite both A$\alpha\beta$ and Aδ afferent fibres during concomitant noxious cutaneous stimulation. To examine the possibility that the nerve stimulation procedures *per se* may have produced irSP release, microprobes were also used to measure irSP release during nerve stimulation alone.

Stimulation of A$\alpha\beta$ Afferent Fibres

As previously presented, noxious mechanical or thermal stimulation of the hindpaw for 15-20 min releases irSP within the region of the substantia gelatinosa. This evoked release was the control situation to compare the effects of peripheral nerve stimulation for any changes in release. Stimulation of tibial A$\alpha\beta$ nerve fibres has previously been shown not to increase the release of irSP in the substantia gelatinosa above basal levels (Duggan & Hendry, 1986) . In

the present study this finding was confirmed for both the tibial and sural nerves, and is consistent with the immunohistochemical localization of irSP in small diameter but not large diameter primary afferent fibres (Hökfelt *et al.* 1975b).

The first series of experiments showed that stimulation of Aαβ fibres had no effect on the irSP release evoked by noxious stimuli. **Fig. 34A** shows the mean image density scan obtained with 29 probes used during noxious heating of the hindpaw for 15 min. **Fig. 34B** shows the mean scan of 29 probes inserted under similar conditions but during continuous tibial nerve stimulation (100 Hz) at Aαβ strength. Both profiles have a zone in which irSP was released reaching a peak at 1.1 mm from the surface of the cord. A minor peak is also visible near the surface of the cord, which may be derived from pial nerve endings (Dalsgaard *et al.* 1982; Risling *et al.* 1984). **Fig. 34A** and **Fig. 34B** are virtually identical, showing no significant differences. Similarly, the release of irSP produced in the substantia gelatinosa region by noxious mechanical stimulation was not significantly altered by concomitant stimulation of sural Aαβ afferent fibres (not illustrated).

Stimulation of Aδ Afferent Fibres

Several studies have shown inhibitory effects of A-afferent stimulation on a C fibre-evoked response of neurones (Chung *et al.* 1984) or of a flexor reflex (Woolf & Wall, 1986a) when the stimulus intensity was sufficient to excite Aδ fibres. Therefore, the amount of noxiously-evoked release of irSP in the presence or absence of Aδ stimulation of the tibial or sural nerve has been determined. Before this study was undertaken, the effects of nerve fibre stimulation at this intensity were determined in the absence of any noxious cutaneous stimuli. **Fig. 35** shows the mean scans for probes inserted in the spinal cord during tibial (A) or sural (B) Aδ nerve fibre stimulation alone. Both procedures released irSP in the region of the substantia gelatinosa. Since sural and tibial Aαβ fibre stimulation produced no significant irSP release above

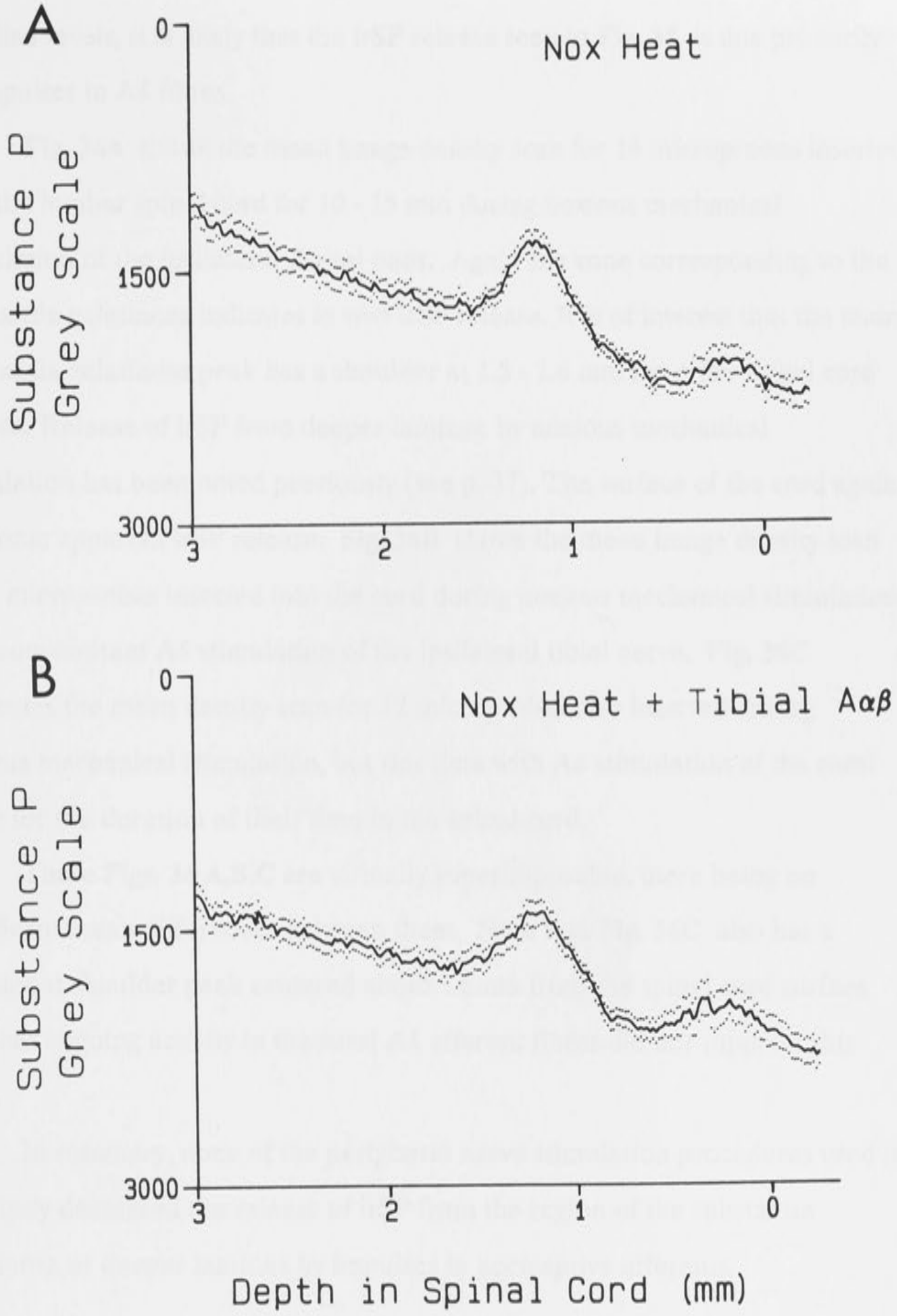


Fig. 34. Release of irSP in the substantia gelatinosa by noxious heat is not inhibited by stimulation of ipsilateral Aαβ afferent fibres of the same spinal segment.

A The mean scan of 29 probes inserted 3.0 mm into the spinal cord during 15 min of noxious heating of the hindpaw (water at 50°C).

B The mean scan of 29 probes treated as in A but with 100 Hz continuous electrical stimulation of the tibial nerve at an intensity sufficient to excite Aαβ afferent fibres.

baseline levels, it is likely that the irSP release seen in **Fig. 35** is due primarily to impulses in Aδ fibres.

Fig. 36A shows the mean image density scan for 14 microprobes inserted into the lumbar spinal cord for 10 - 15 min during noxious mechanical stimulation of the ipsilateral digital pads. Again the zone corresponding to the substantia gelatinosa indicates *in vivo* irSP release. It is of interest that the main substantia gelatinosa peak has a shoulder at 1.5 - 1.6 mm from the spinal cord surface. Release of irSP from deeper laminae by noxious mechanical stimulation has been noted previously (see p. 37). The surface of the cord again has some apparent irSP release. **Fig. 36B** shows the mean image density scan of 12 microprobes inserted into the cord during noxious mechanical stimulation with concomitant Aδ stimulation of the ipsilateral tibial nerve. **Fig. 36C** illustrates the mean density scan for 12 microprobes also inserted during noxious mechanical stimulation, but this time with Aδ stimulation of the sural nerve for the duration of their time in the spinal cord.

These **Figs. 36 A,B,C** are virtually superimposable, there being no significant mean differences between them. Note that **Fig. 36C** also has a prominent shoulder peak centered about 1.6mm from the spinal cord surface and that ongoing activity in the sural Aδ afferent fibres did not suppress this peak.

In summary, none of the peripheral nerve stimulation procedures used in this study decreased the release of irSP from the region of the substantia gelatinosa or deeper laminae by impulses in nociceptive afferents.

Discussion

Impulses in primary afferent fibres to the spinal cord may excite dorsal horn neurones or inhibit them indirectly. The excitation produced by a C fibre input can be inhibited by impulses in A fibres of the tibial nerve (Cervero *et al.* 1976) and of the sural nerve (Gregor & Zimmerman, 1972) of the cat

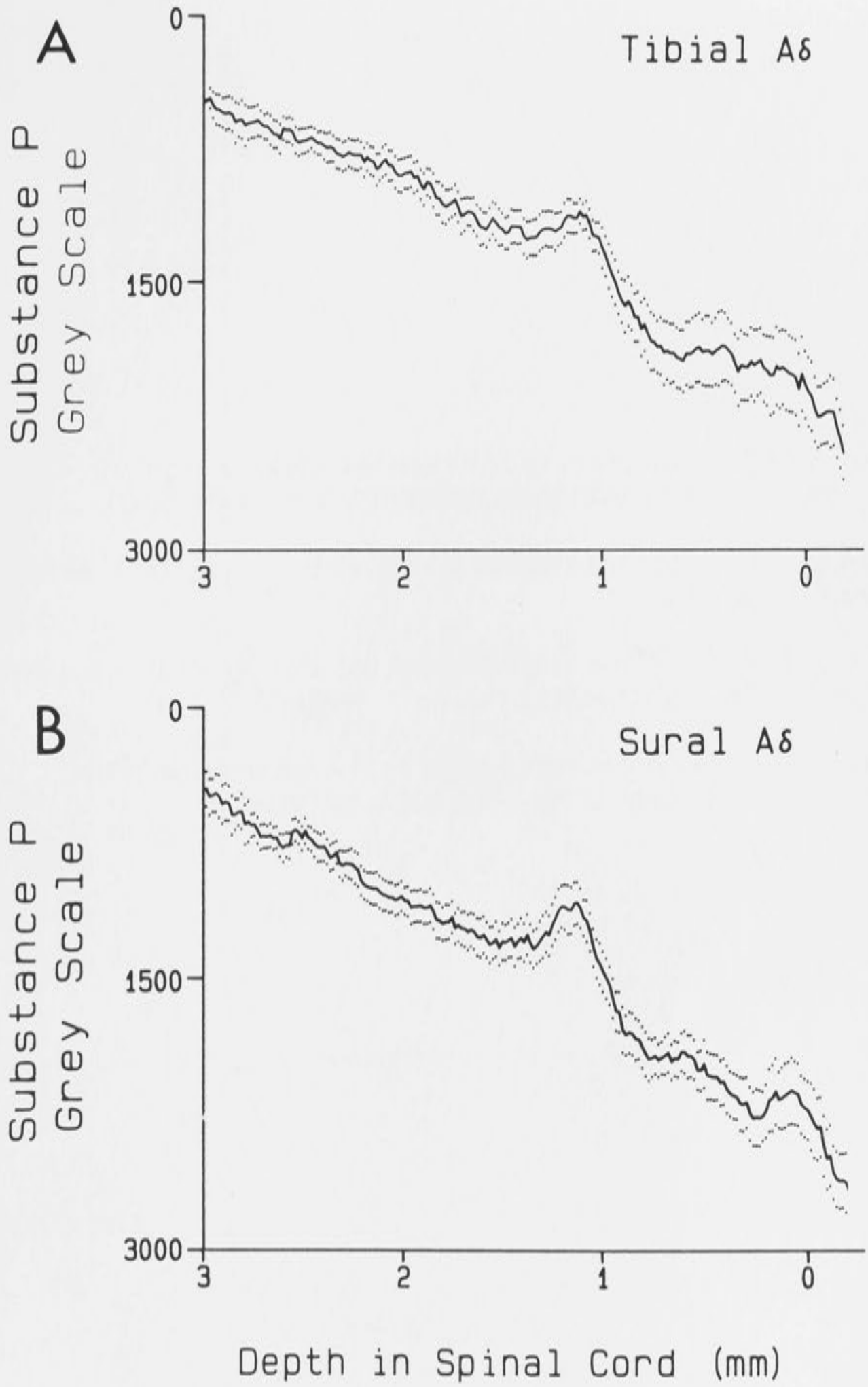


Fig. 35. Stimulation at intensities sufficient to excite Aδ afferent fibres results in release of irSP in the substantia gelatinosa of the spinal cord.

A Mean scan of 12 microprobes inserted during electrical stimulation of Aδ afferent fibres of the tibial nerve.

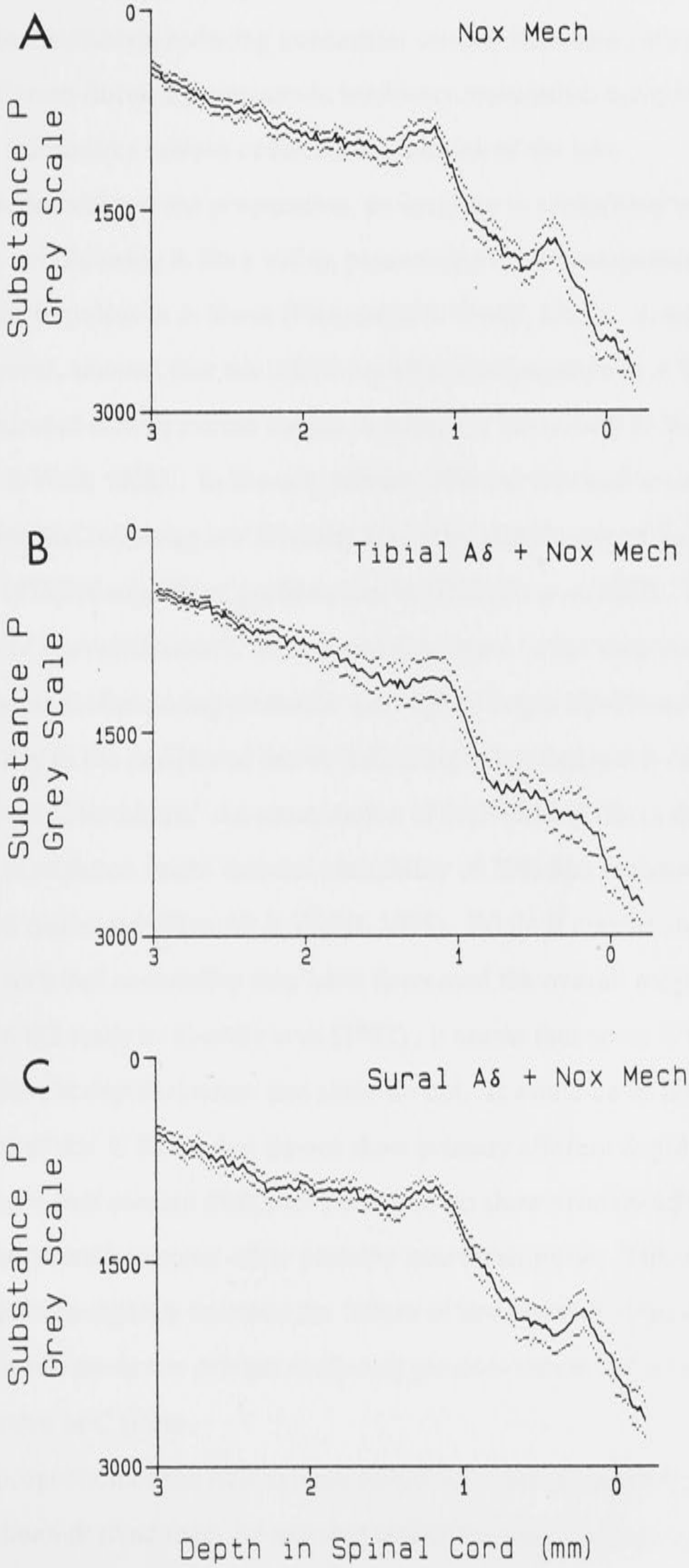
B Mean scan of 12 microprobes inserted during electrical stimulation of Aδ afferent fibres of the sural nerve.

Fig. 36. Lack of segmental control of irSP release evoked by noxious mechanical stimulation in the substantia gelatinosa of the spinal cord.

A Mean scan of 12 microprobes inserted during noxious mechanical stimulation of the ipsilateral digital pads.

B Mean scan of 12 microprobes treated as in A but with electrical stimulation of Aδ afferent fibres of the ipsilateral tibial nerve.

C Mean scan of 12 microprobes with treated as in A but with electrical stimulation of Aδ afferent fibres of the ipsilateral sural nerve.


A
Nox Mech
B
Tibial Aδ + Nox Mech
C
Sural Aδ + Nox Mech
Substance P
Grey Scale
0
1500
3000
3
2
1
0
Depth in Spinal Cord (mm)

hindlimb. There is controversy as to whether this inhibition is mediated by a presynaptic mechanism reducing transmitter release from unmyelinated primary afferent fibres, a postsynaptic inhibitory mechanism independent of excitatory transmitter release or some combination of the two.

In a decerebrate rat preparation, an increase in excitability of C fibres followed a conditioning A fibre volley, presumably due to a depolarization of C-terminals by impulses in A fibres (Fitzgerald & Woolf, 1981) . A subsequent study, however, showed that the inhibitory effects of impulses in A fibres on C-evoked neuronal activity occurs mainly in lamina V and rarely in laminae I and II (Woolf & Wall, 1982) . In the cat, primary afferent depolarization of C fibres has been studied following low intensity electrical stimulation of the tibial nerve (Calvillo, 1978) or superficial peroneal nerve (Calvillo *et al.* 1982) . The amplitude of the antidromic C fibre population spike in the sural nerve set up by an intraspinal stimulating electrode was slightly larger 30-40 msec after an A fibre volley in the peripheral nerve, indicating a depolarization of a population of C-terminals. An examination of individual C fibres during low threshold stimulation found that the excitability of 20% was increased and that of 26% was decreased (Hentall & Fields, 1979). While it may be that the mixed effects on terminal excitability may have decreased the overall magnitude of the response in the study by Calvillo *et al.* (1982) , it seems that some C fibres show primary afferent depolarization and some do not. It would be of interest to know whether the C fibres that do not show primary afferent depolarization are also those that contain irSP, and those that do show primary afferent depolarization contain some other putative neurotransmitter. This may explain the apparent discrepancy between the failure of low intensity stimulation to inhibit irSP release in the present study and previous reports of primary afferent depolarization of C fibres.

A proportion of the irSP release observed in the present experiments may have been derived from Aδ afferent terminals, and it is possible that such

release may be subject to presynaptic inhibition. Sastry (1978) has reported excitability increases in Aδ terminals by electrical stimulation of low threshold cutaneous afferents. Regardless of the origin of the irSP release observed here, however, it was not reduced by primary afferent stimulation.

Since axoaxonic synapses are rare or absent on primary afferent terminals (see p. 70-71), a more likely explanation of the present results is that inhibition of nociceptive responses by activity in large diameter fibres is a post-synaptic event. Early work showed disynaptic IPSP's in spinocervical tract neurones following stimulation of high threshold cutaneous afferent fibres suggesting postsynaptic inhibition of spinal transmission cells (Hongo *et al.* 1968). Other work is suggestive of a postsynaptic inhibitory process acting directly on the second order neurone. In laminae V-VII of the cat lumbar spinal cord, the excitation of some interneurones by microelectrophoretic glutamate could be partially suppressed by peripheral nerve stimulation (Morris, 1987) or by natural stimulation of the segmental inhibitory field (Besson *et al.* 1974) .

Anatomical studies of second order neurones in the substantia gelatinosa receiving nociceptive input have revealed neurones with projections to deeper laminae (Light & Kavookjian, 1988). Inhibitory synapses were illustrated at the initial segment of the myelinated axon projecting out of the substantia gelatinosa and it was suggested this could subserve post-synaptic inhibition of this nociceptive neurone. It remains to be determined how prevalent these structures are in the upper dorsal horn. The current anatomical evidence seems to support our findings of an absence of a reduction in irSP release by stimulation of A-afferent fibres of the tibial or sural nerves.

The observation of irSP release from deeper laminae might be explained by a functional specificity in the termination sites of the cutaneous nociceptors. The nociceptors responding to heat may terminate in laminae I and II exclusively whereas the nociceptors responding to mechanical stimulation may

have their terminals in laminae V-VI as well as in laminae I and II. Although a population of cutaneous mechano-nociceptors have been described which respond only to noxious mechanical stimulation and not noxious thermal or chemical stimulation (Burgess & Perl, 1967) , the large majority of small diameter afferent fibres are polymodal nociceptors responding to all three forms of stimulation. Regardless of the origins of this deeper zone of release, it seems clear that the release of irSP is still present, if not larger, with the electrical stimulation of the higher threshold A-afferent fibres of the sural nerve.

It is possible that in addition to SP, glutamate is also released in the spinal cord by noxious cutaneous stimulation. In fact, glutamate and SP coexist in small dorsal root ganglion neurons (Battaglia & Rustioni, 1988) and have been localized in separate vesicles within terminals of small diameter fibres (De Biasi & Rustioni, 1988). A selective presynaptic control of release of individual vesicle types has not been demonstrated, and it is probable that inhibition of SP release in the substantia gelatinosa reflects presynaptic control of nociceptive transmission. The major finding of the present study is that electrical stimulation of tibial or sural A-afferent fibres does not produce an inhibition of irSP released from the terminals of C fibres activated by noxious stimuli. This evidence does not support the notion that the analgesic effects of low threshold nerve stimulation are due to a presynaptic inhibition of SP release.

6. PHARMACOLOGICAL CONTROL OF SENSORY NEUROPEPTIDE RELEASE

Acute Morphine and SP, CGRP release

Introduction

Morphine administered systemically or by microelectrophoresis into the substantia gelatinosa inhibits the responses of dorsal horn neurones to noxious stimuli (Duggan *et al.* 1977a). The implication of SP as a nociceptive transmitter of primary afferent fibres (see **General Introduction**) gives rise to the hypothesis that the analgesic action of morphine at the level of the spinal cord may be mediated by a decrease in the release of SP in the substantia gelatinosa.

There is evidence from *in vitro* and *in vivo* studies that morphine and other opioids may act to reduce the noxiously-evoked release of SP in the spinal cord. Several opioids were found to inhibit the potassium-induced release of irSP into perfusates of rat trigeminal nucleus slices (Jessell & Iversen, 1977), and macerated rat spinal cord (Pang & Vasko, 1986). Further work *in vivo* showed that morphine (0.1 - 10 μM in the perfusate) decreases the release of irSP in a naloxone-reversible manner, into spinal superfusates of the cat spinal cord (Yaksh *et al.* 1980; Go & Yaksh, 1987). In the rabbit with a push-pull cannula inserted into the dorsal horn, the release of irSP evoked by noxious mechanical stimulation was inhibited by a high dose (10 mg/kg) of morphine, an effect which showed partial reversal with naloxone. However, a lower dose of morphine which was still analgesic (1 mg/kg) did not decrease irSP release (Hirota *et al.* 1985). Morphine and met-enkephalin (10 μM) circulating in the push-pull perfusion system, inhibited the noxiously-evoked release of irSP, but dynorphin A was ineffective (Hirota *et al.* 1985). A similar result was found with irSP sampled by push-pull perfusion of the trigeminal nucleus caudalis (Yonehara *et al.* 1988), where morphine given systemically (10 mg/kg) or locally

(10 μM in the perfusate) inhibited the irSP release evoked by tooth pulp stimulation.

These studies are supported by electrophysiological findings using the indirect method of measuring terminal excitability through a decrease in antidromic threshold (Wall, 1958). Presynaptic depolarization by microelectrophoretically administered morphine was found in more than half of the finely myelinated Aδ fibres studied (Carstens *et al.* 1979), and systemic opioids enhanced the depolarization of C fibres by impulses in large diameter cutaneous afferents (Sastry, 1979).

The density of opiate binding sites in the substantia gelatinosa has been reported to decrease following the degeneration of small diameter fibres in the dorsal roots, a finding which suggests the presence of opiate receptors on primary afferent fibres (Fields *et al.* 1980). Furthermore, endogenous opioids such as enkephalin and dynorphin are found in the substantia gelatinosa (Hökfelt *et al.* 1977; Hunt *et al.* 1980; Miller & Seybold, 1987, 1988), giving a physiological basis to the observed pharmacology.

The present experiments were designed to test whether or not morphine reduces the intraspinal release of two neuropeptides, SP or CGRP, in the region of the substantia gelatinosa as detected by antibody microprobes.

Results

Release of irSP after acute morphine

The release of irSP in the substantia gelatinosa shown in **Fig. 37** was evoked with either noxious thermal, mechanical cutaneous or electrical nerve stimuli. Following the epipial superfusion of the spinal cord with a 2, 2.5, or 5 X 10^{-3}M morphine in Ringer's solution, there was no significant difference in the noxiously-evoked irSP release (difference scan and t-test not shown). In addition, the systemic administration of morphine over a large dose range (1 -

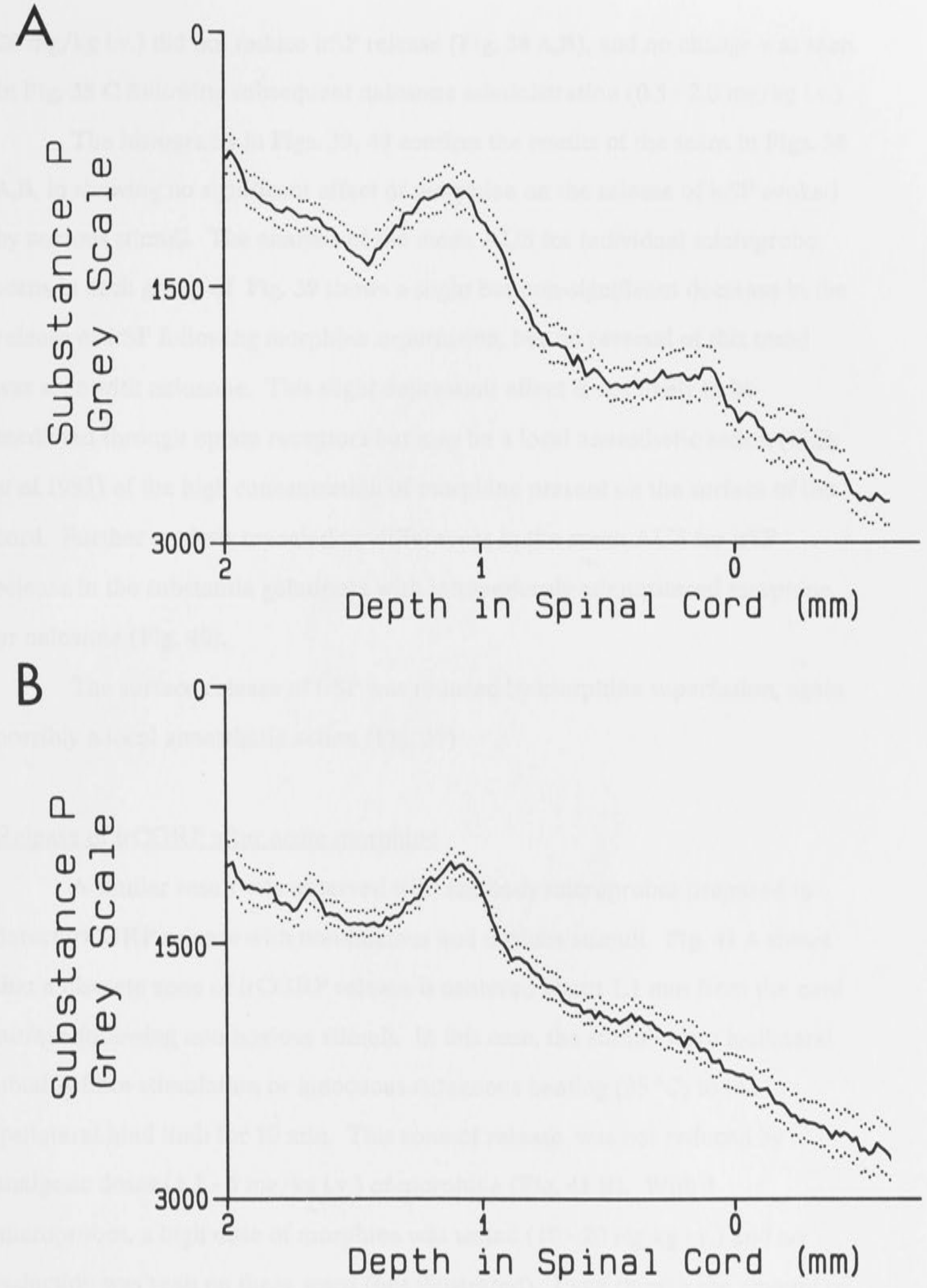


Fig. 37. Morphine superfusion does not reduce the release of irSP in the cat substantia gelatinosa. Noxious heating or mechanical stimulation of the digital pads or electrical stimulation of the tibial nerve at C fibre threshold was applied for 15, 20 or 30 min ipsilateral to the placement of microprobes. Microprobes in this series were inserted to a depth of 2.0 mm.

A. Pre-morphine controls (N = 30).

B. Post-morphine superfusion (N = 44) with 2, 2.5 or 5 x 10^{-3}M morphine HCl.

20 mg/kg i.v.) did not reduce irSP release (**Fig. 38 A,B**), and no change was seen in **Fig. 38 C** following subsequent naloxone administration (0.5 - 2.0 mg/kg i.v.).

The histograms in **Figs. 39, 40** confirm the results of the scans in **Figs. 38 A,B**, in showing no significant effect of morphine on the release of irSP evoked by noxious stimuli. The analysis of the mean AUS for individual microprobe scans in each group of **Fig. 39** shows a slight but non-significant decrease in the release of irSP following morphine superfusion, but no reversal of this trend was seen with naloxone. This slight depressant effect is not likely to be mediated through opiate receptors but may be a local anaesthetic action (Gilly *et al.* 1985) of the high concentration of morphine present on the surface of the cord. Further analysis revealed no differences in the mean AUS for irSP release in the substantia gelatinosa with intravenously administered morphine or naloxone (**Fig. 40**).

The surface release of irSP was reduced by morphine superfusion, again possibly a local anaesthetic action (**Fig. 37**)

Release of irCGRP after acute morphine

A similar result was observed with antibody microprobes prepared to detect irCGRP release with non-noxious and noxious stimuli. **Fig. 41 A** shows that a discrete zone of irCGRP release is centered about 1.1 mm from the cord surface following non-noxious stimuli. In this case, the stimuli were ipsilateral tibial A fibre stimulation or innocuous cutaneous heating (35 °C) to the ipsilateral hind limb for 10 min. This zone of release was not reduced by analgesic doses (1.1 - 5 mg/kg i.v.) of morphine (**Fig. 41 B**). With 3 microprobes, a high dose of morphine was tested (10 - 20 mg.kg i.v.) and no reduction was seen on these scans (not illustrated). Even though the amount of irCGRP release appears to be slightly larger in **Fig. 41 B**, the substraction of the two mean scans in **Fig. 41 C** produced no significant differences (not shown) in the region of the substantia gelatinosa.

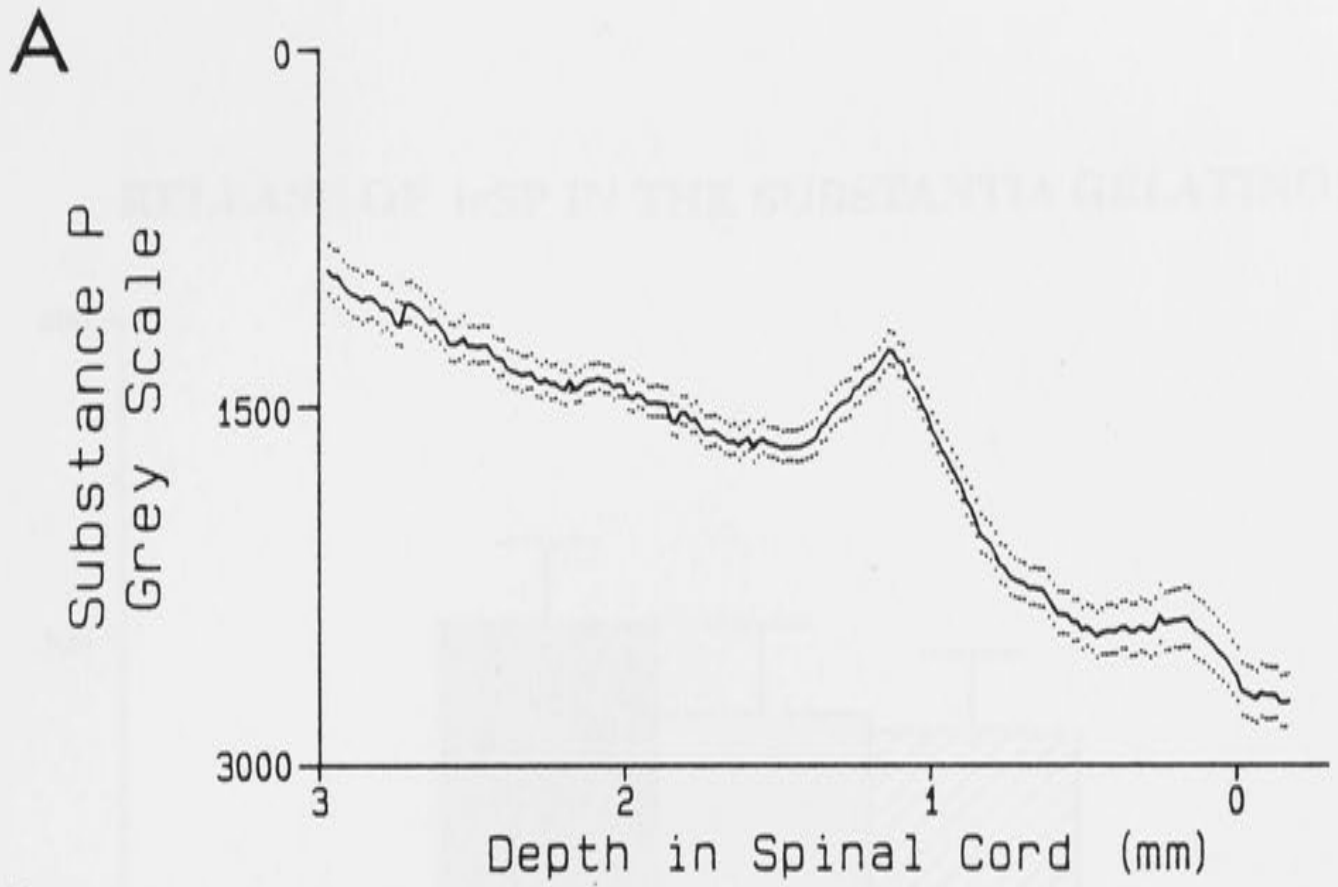
Fig. 38. No effect of systemic morphine on the release of irSP in the substantia gelatinosa.

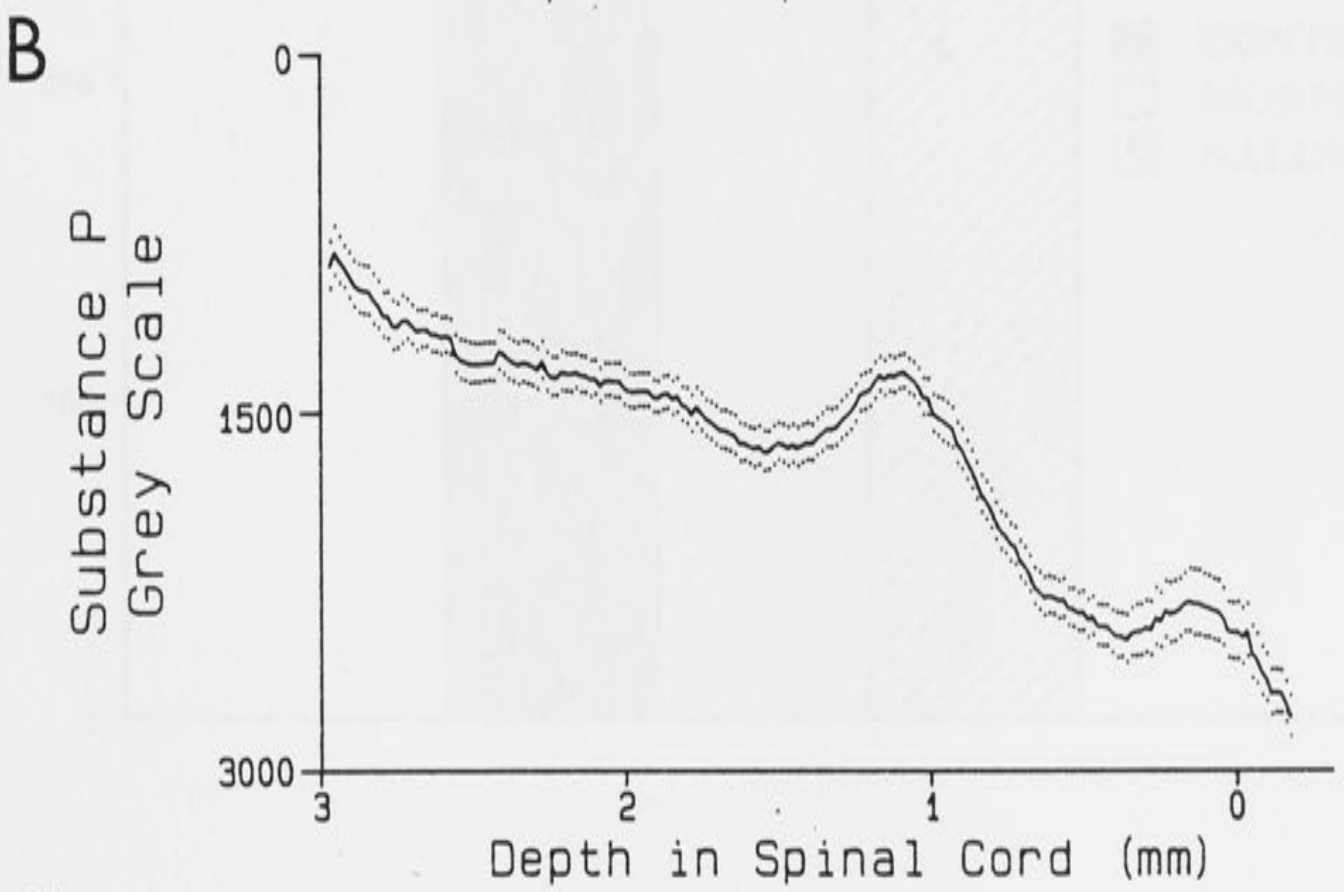
All stimuli and times as in **Fig. 37**.

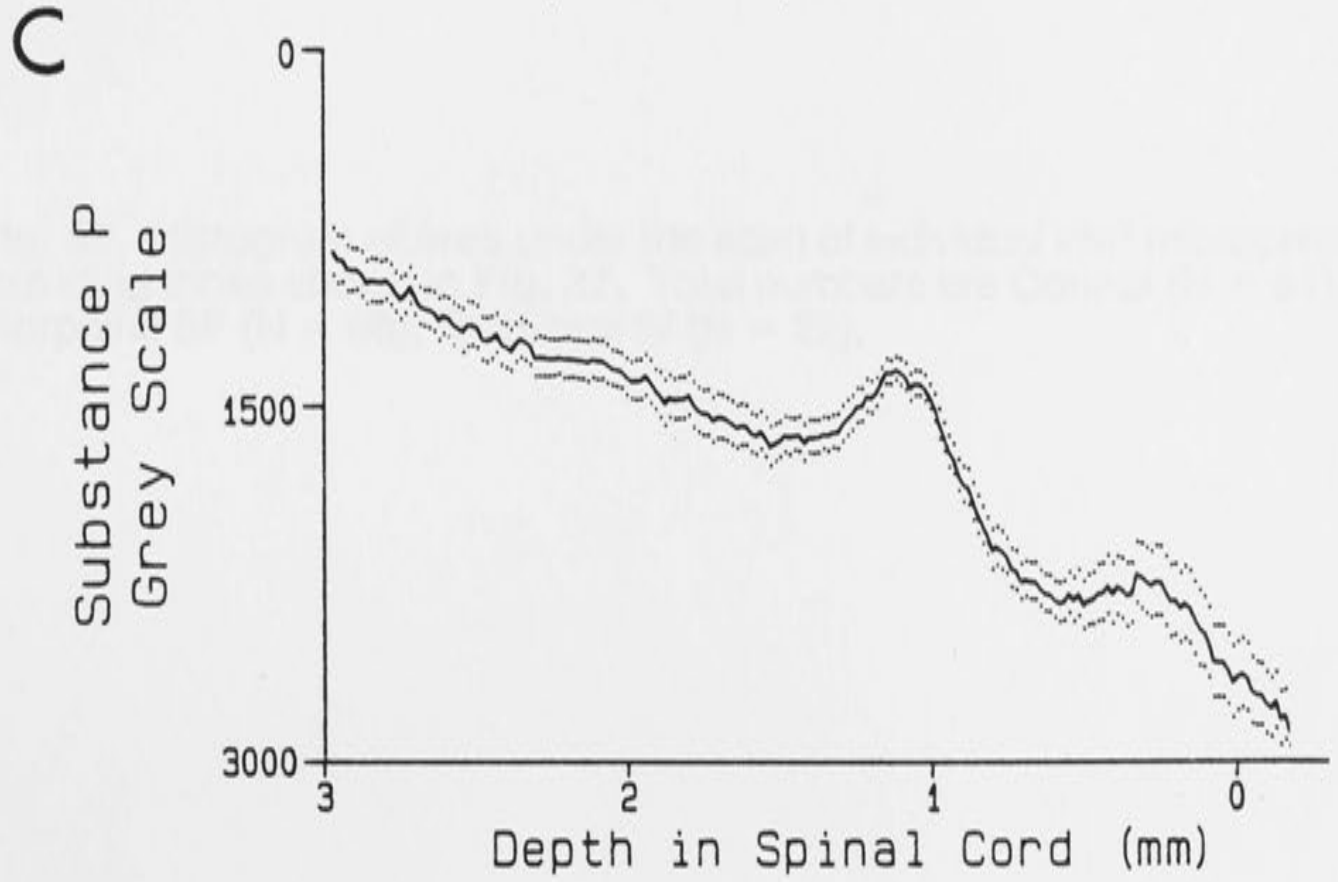
A. Pre-morphine controls (N = 33).

B. Post-morphine HCl i.v., 1 - 20 mg/kg (N = 38)

C. Post-naloxone i.v., 0.5 - 2 mg/kg (N = 24)


A
Substance P
Grey Scale
0
1500
3000
3
2
1
0
Depth in Spinal Cord (mm)


B
Substance P
Grey Scale
0
1500
3000
3
2
1
0
Depth in Spinal Cord (mm)


C
Substance P
Grey Scale
0
1500
3000
3
2
1
0
Depth in Spinal Cord (mm)

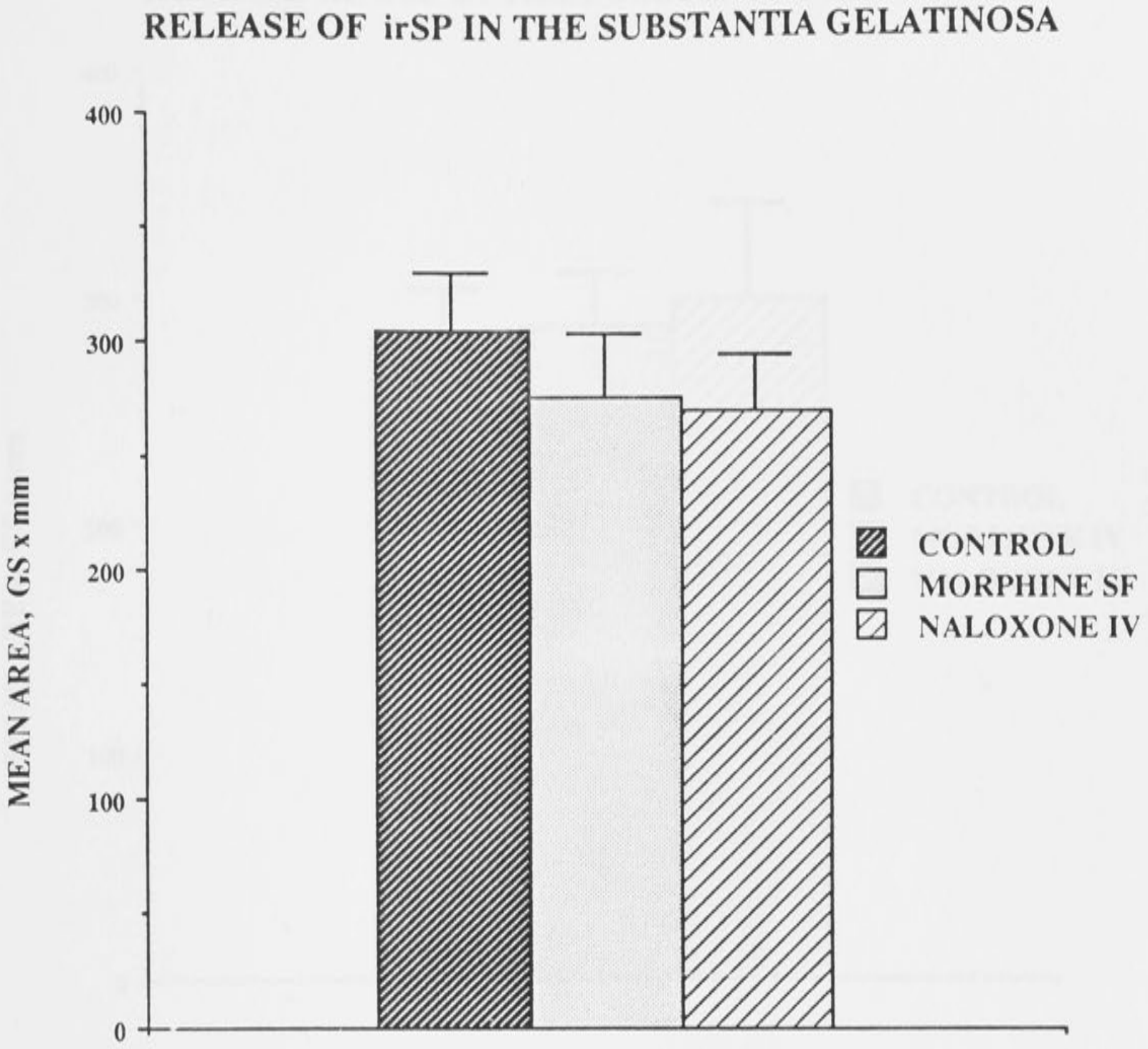


Fig. 39. Histogram of area under the scan of individual irSP microprobes including those shown in **Fig. 37.** Total numbers are Control (N = 51), Morphine SF (N = 66), Naloxone IV (N = 22).

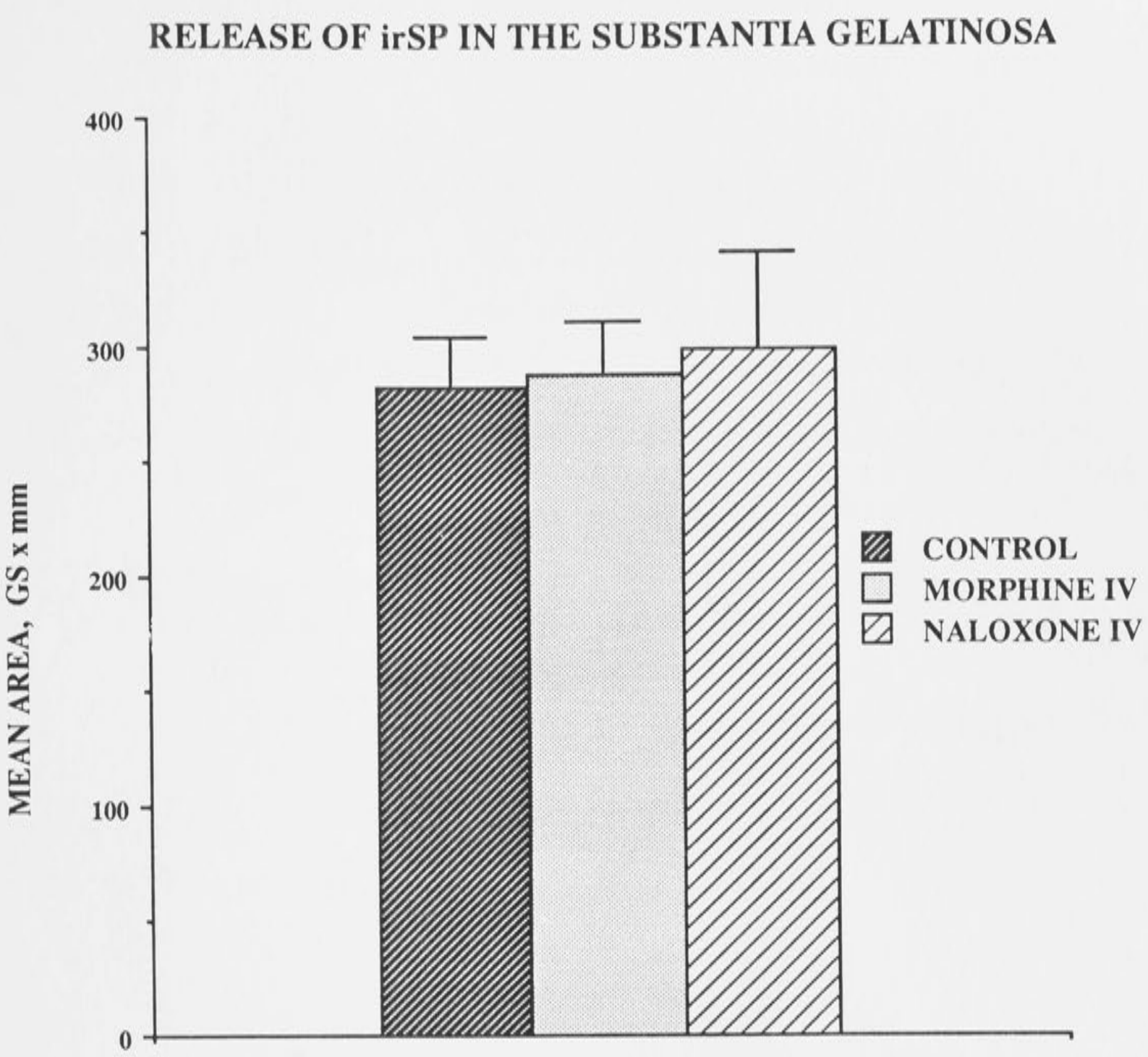


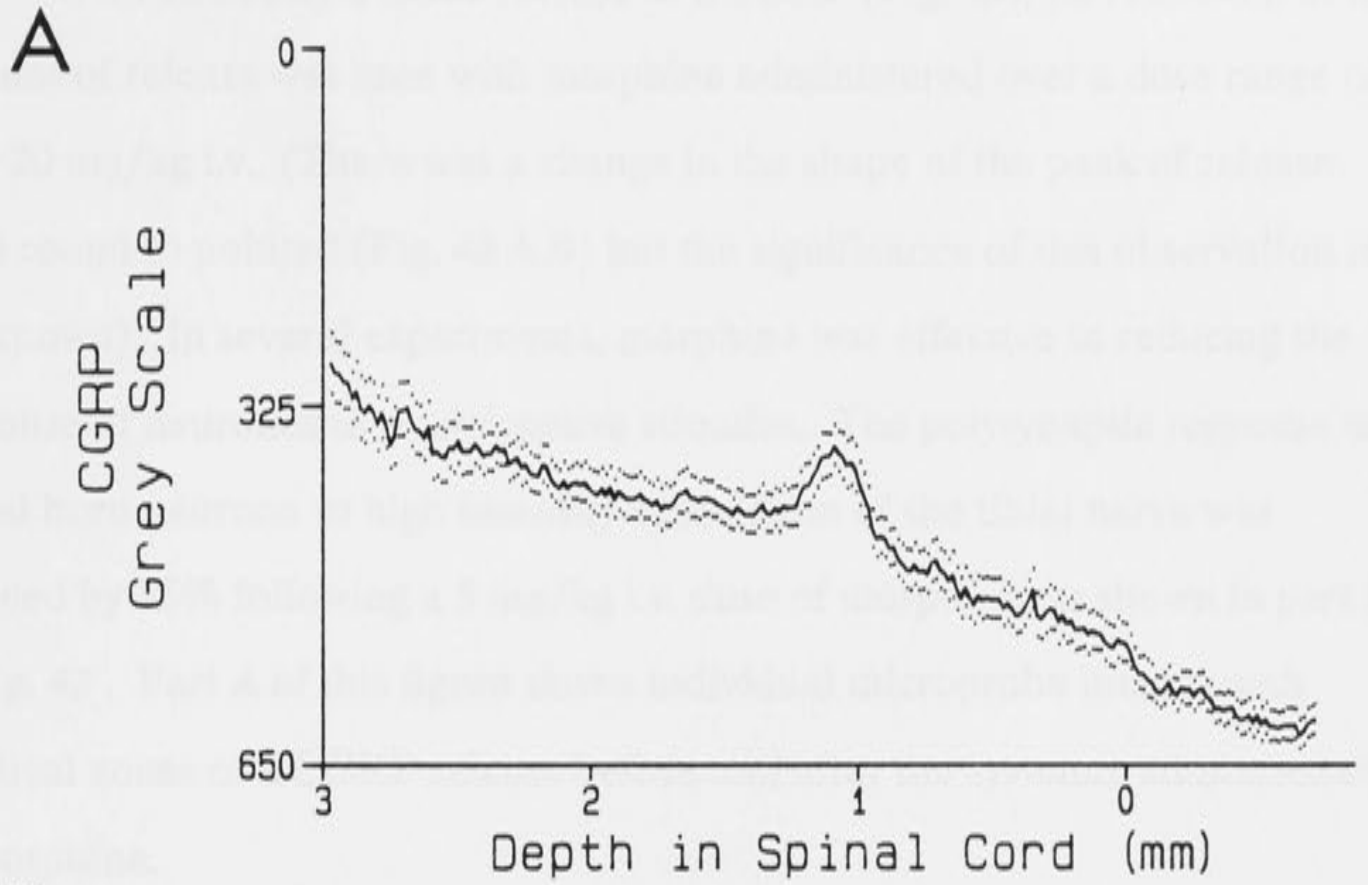
Fig. 40. Histogram of area under the scan of individual irSP microprobes including those shown in **Fig. 38.** Total numbers are Control (N = 59), Morphine IV (N = 56), Naloxone IV (N = 28).

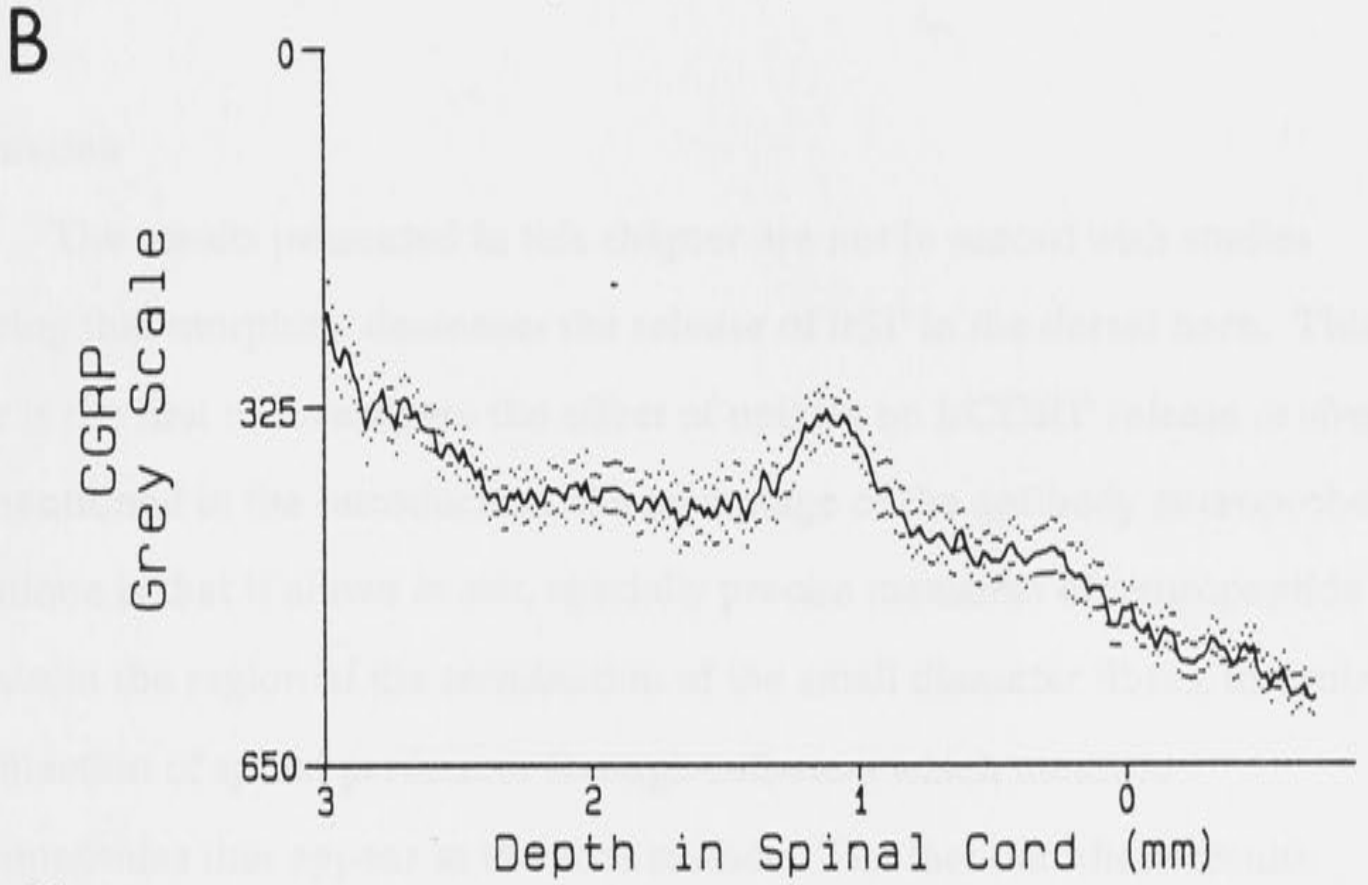
Fig. 41. Acute morphine does not inhibit basal irCGRP release.

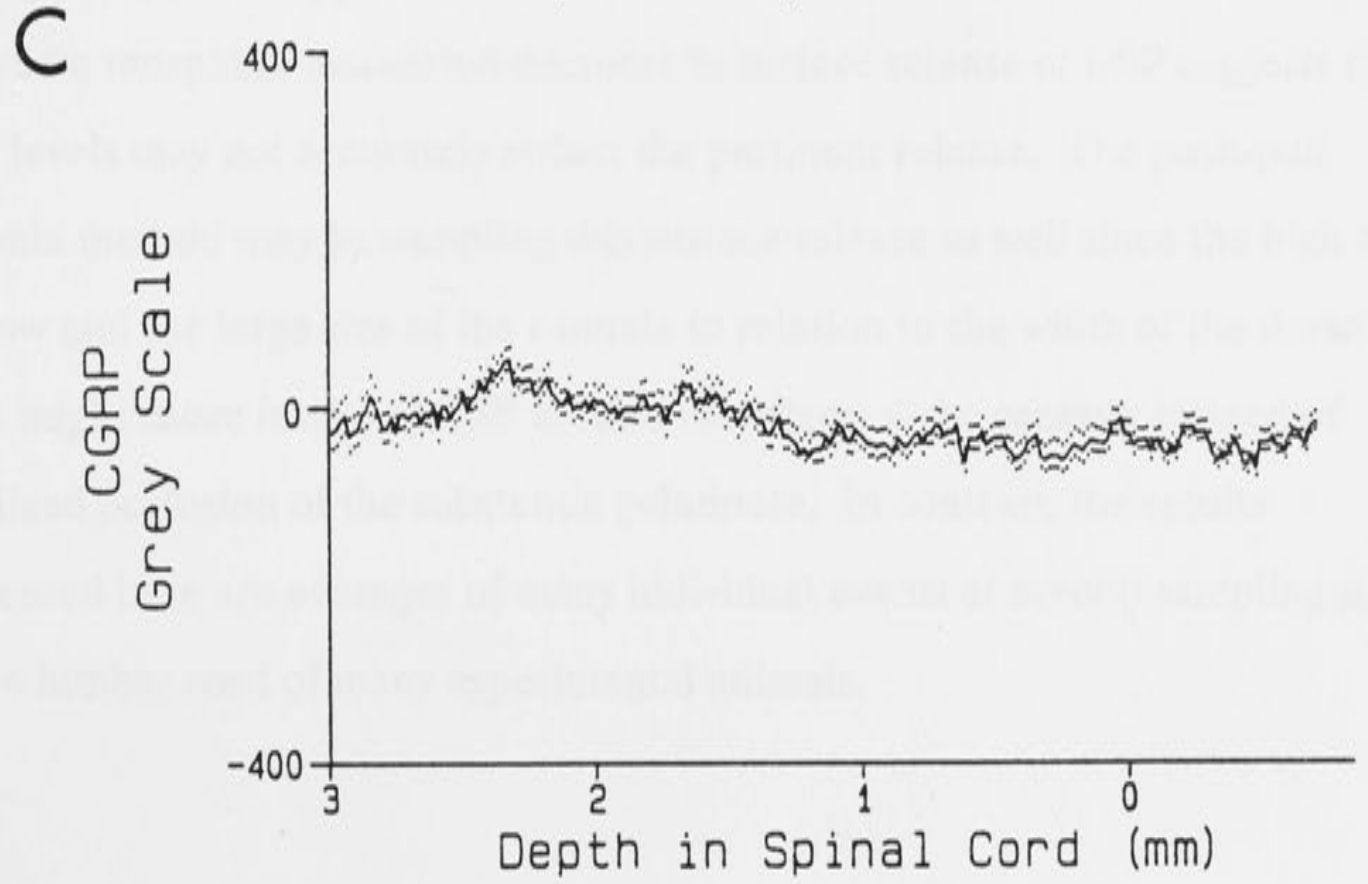
A. Pre-Morphine: Mean scan of 27 microprobes inserted during non-noxious stimulation of the ipsilateral hind limb with 35^{O}C water (N = 17) or tibial nerve at 1.5 x T (N = 10) for 10 min.

B. Post-Morphine: Mean scan of 28 microprobes inserted during stimuli identical to **A**, but following 1.1 - 5 mg/kg (N = 13) or 10 - 20 mg/kg (N = 3) morphine HCl.

C. The difference between the scans shown in A and B.


A
0
325
650
CGRP
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)


B
0
325
650
CGRP
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)


C
400
0
-400
CGRP
Grey Scale
3
2
1
0
Depth in Spinal Cord (mm)

With noxiously-evoked release of irCGRP (**Fig. 42**), no reduction in the amount of release was seen with morphine administered over a dose range of 5.0 - 20 mg/kg i.v. (There was a change in the shape of the peak of release from round to pointed (**Fig. 42 A,B**) but the significance of this observation is not known). In several experiments, morphine was effective in reducing the response of neurones to a nociceptive stimulus. The polysynaptic response of a dorsal horn neurone to high intensity stimulation of the tibial nerve was reduced by 35% following a 5 mg/kg i.v. dose of morphine, as shown in part **B** of **Fig. 43** . Part **A** of this figure shows individual microprobe images with identical zones of irCGRP release before and after the systemic administration of morphine.

Discussion

The results presented in this chapter are not in accord with studies showing that morphine decreases the release of irSP in the dorsal horn. This work is the first to investigate the effect of opiates on irCGRP release *in vivo*. As mentioned in the introduction, the advantage of the antibody microprobe technique is that it allows *in situ*, spacially precise measures of neuropeptide release in the region of the termination of the small diameter fibres, in contrast to collection of spinal perfusates through catheters which measure neuropeptides that appear at the cord surface. The fact that these results showed a morphine-associated decrease in surface release of irSP suggests that CSF levels may not accurately reflect the pertinent release. The push-pull cannula method may be sampling this surface release as well since the high rate of flow and the large size of the cannula in relation to the width of the dorsal horn might cause intake of CSF around the edges of the cannula instead of localized perfusion of the substantia gelatinosa. In contrast, the results presented here are averages of many individual events at several sampling sites in the lumbar cord of many experimental animals.

Fig. 42. Acute morphine does not inhibit noxiously-evoked irCGRP release.

A. Pre-Morphine: Mean scan of 30 microprobes inserted during noxious stimulation of the ipsilateral hind limb with 52^{o}C water (N = 18) or tibial nerve at 200-300 x T (N = 12) for 10 min.

B. Post-Morphine: Mean scan of 33 microprobes inserted during stimuli identical to **A**, but following 5.1 - 9.9 mg/kg (N = 15) or 10 - 20 mg/kg (N = 18) morphine HCl.

C. The difference between the scans shown in A and B.

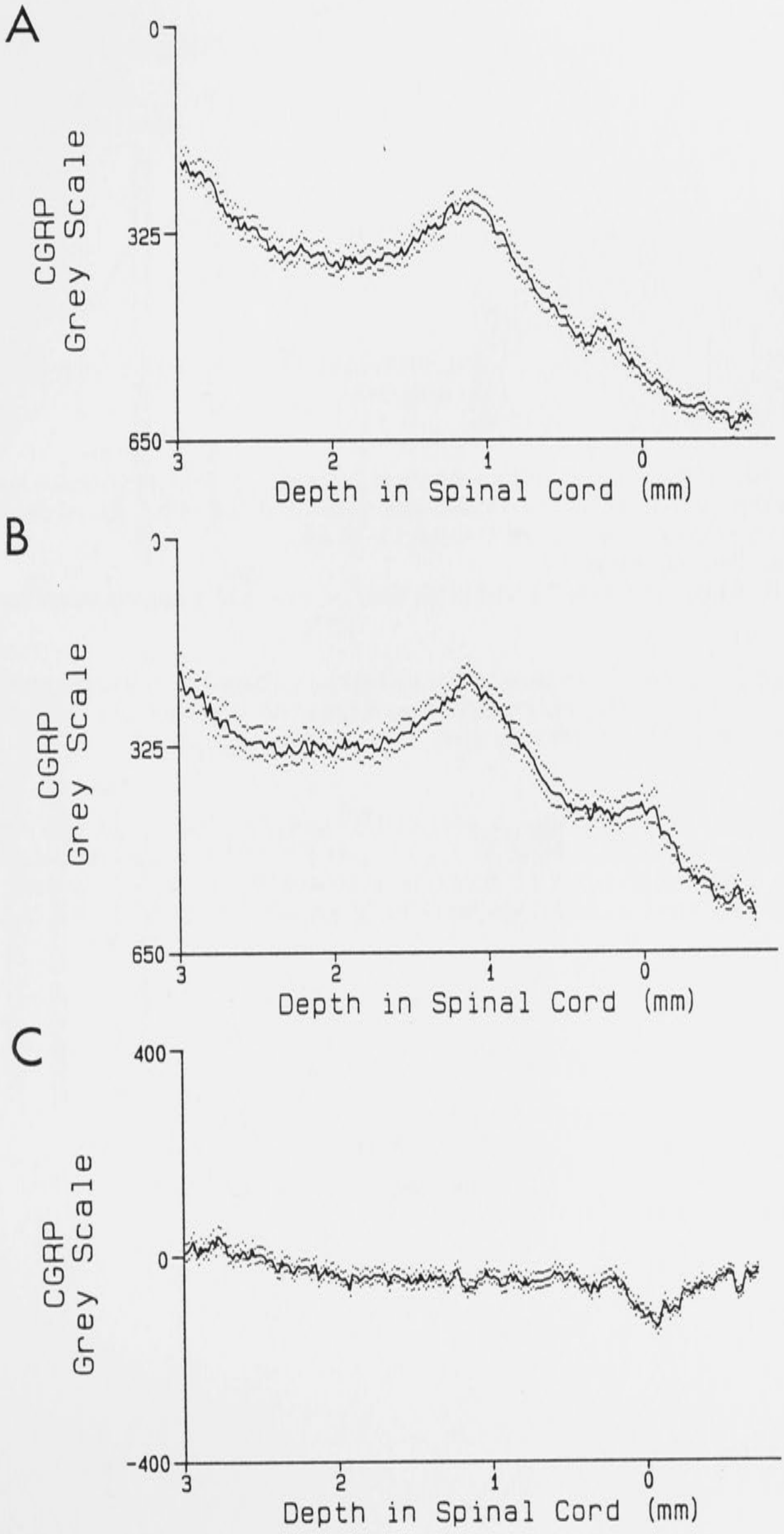

A
CGRP
Grey Scale
0
325
650
3
2
1
0
Depth in Spinal Cord (mm)
B
CGRP
Grey Scale
325
650
3
2
1
0
Depth in Spinal Cord (mm)
C
CGRP
Grey Scale
400
0
-400
3
2
1
0
Depth in Spinal Cord (mm)

Fig. 43. The analgesic action of morphine does not result from a presynaptic inhibition of release of sensory neuropeptides.

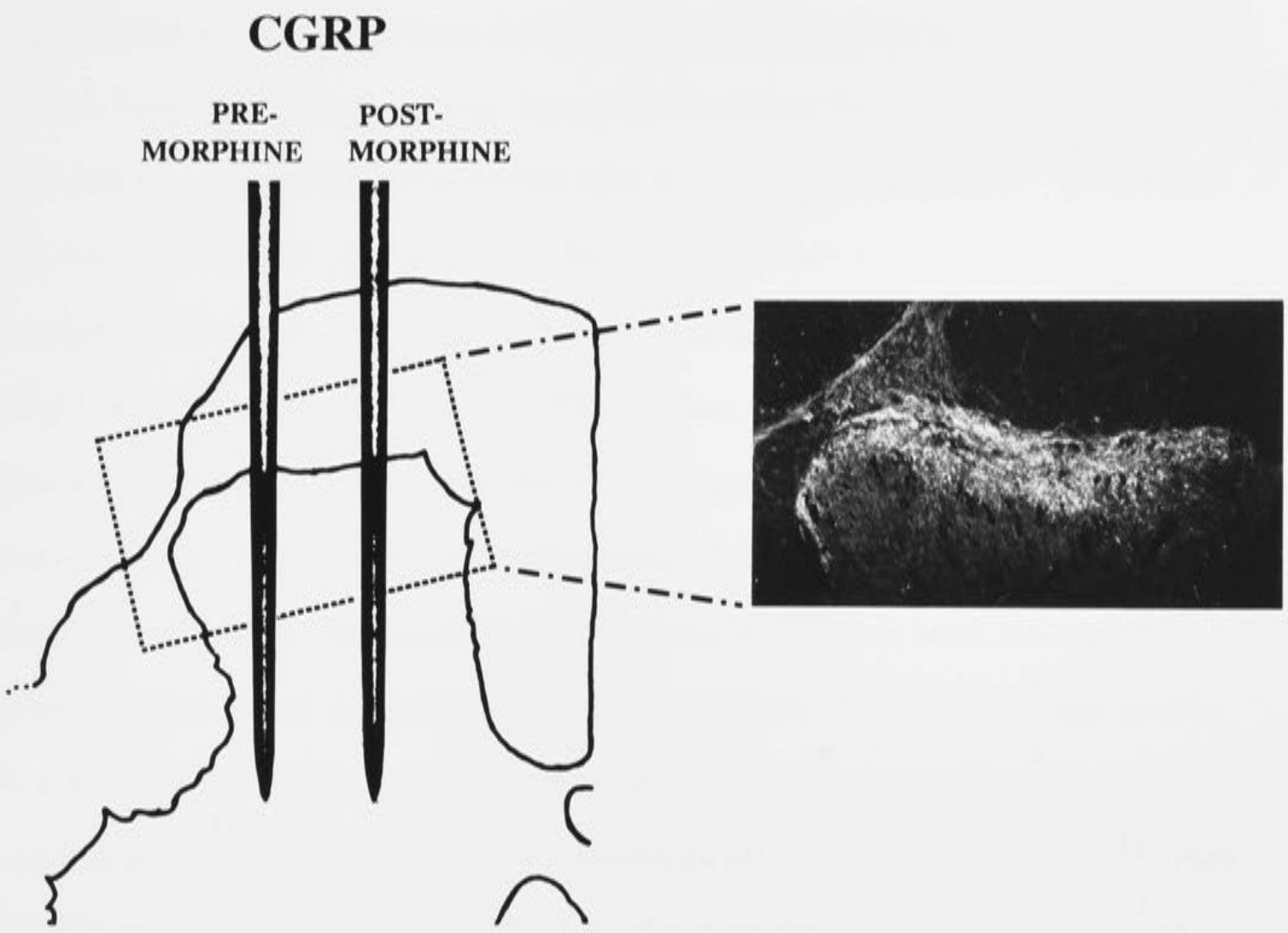
A. Left: Photographic enlargements of autoradiographic microprobe images arranged on a line drawing of a transverse section of the lumbar spinal cord. Stimuli for both probes was 52^{O}C water for 10 min.

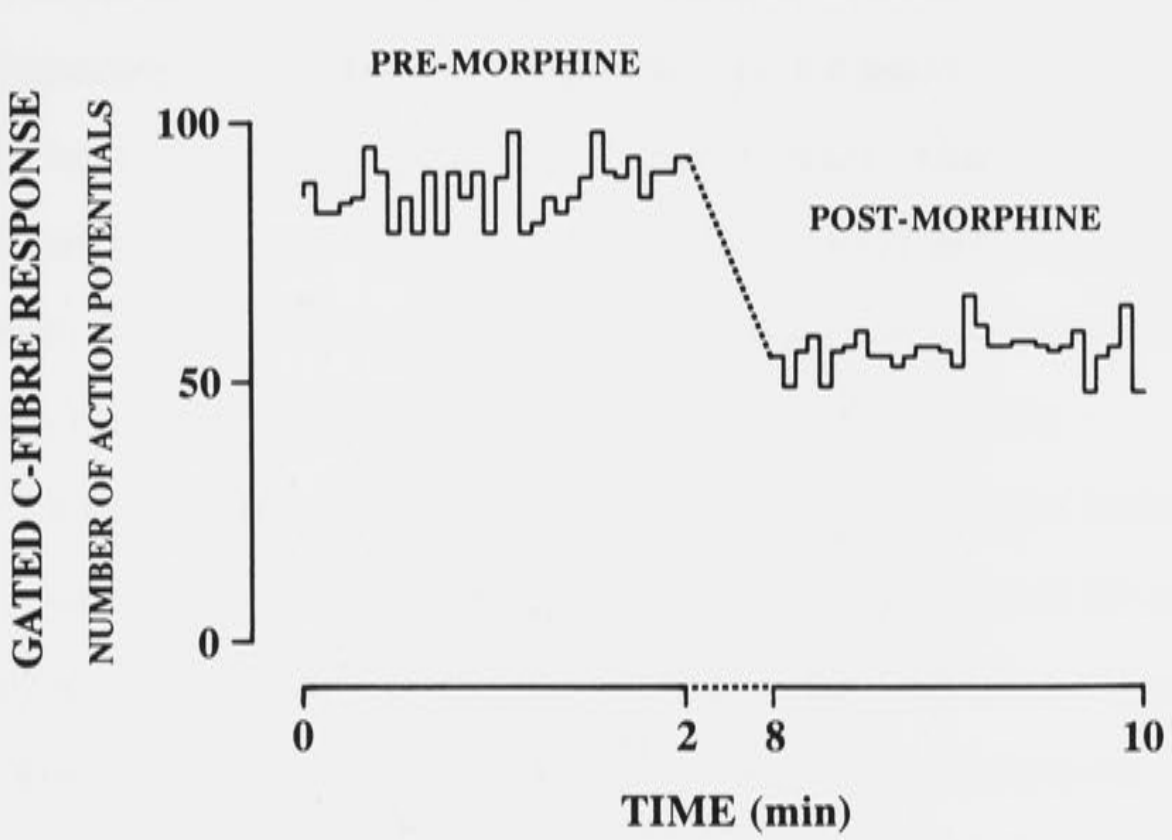
A. Pre-morphine.

B. Post-morphine, 7 and 81 min after i.v. doses of 5 mg/kg morphine HCl.

Right: Immunofluorescence histochemistry (Texas Red- Streptavidin) of irCGRP in the dorsal horn of the spinal cord detected with the same antibody present on the antibody microprobes. Photo magnification x 45.

B. Gated C-fibre response of a dorsal horn interneurone (depth 1.94 mm) to stimulation of the tibial nerve at 20 V, 2 x 0.5 ms pulses 3 ms apart repeated at 0.28 Hz. Pre-morphine gated C fibre response was 88.7 ± 5.7 (S.D.) action potentials per stimulus and post-morphine (5 mg/kg i.v.) was 57.3 ± 4.2 (S.D.).


CGRP
PRE-
MORPHINE
POST-
MORPHINE


PRE-MORPHINE
POST-MORPHINE
GATED C-FIBRE RESPONSE
NUMBER OF ACTION POTENTIALS
100
50
0
0
2
8
10
TIME (min)

The electrophysiological studies of inhibition by opioids of small diameter primary afferents have not yielded consistent results. Threshold increases have been observed (i.e. *hyperpolarized* terminals), and few C fibres (2/6) have shown naloxone-reversible effects (Carstens *et al.* 1979). In another study, meperidine (0.2 mg/kg i.v.) increased the threshold for antidromic activation of all C fibres investigated (Sastry, 1980). As outlined by Duggan & North, 1983, there are a number of different interpretation of these findings: 1) an increase in excitability may be due to the suppression by morphine of a hyperpolarizing effect of inhibitory interneurones presynaptic to the terminal without any effect on the terminal *per se*; 2) an increase in terminal excitability may be due to a direct depolarization of the terminal by morphine associated with a increase in conductance, which would tend to shunt the incoming action potentials and the effect of inhibitory feedback on it, etc. However, all of these mechanisms could produce an inhibition of transmitter release and the more serious consideration is that changes in terminal threshold may simply result from movement of the tip of the electrode used for antidromic stimulation away from the terminal with the changes in blood pressure brought on by systemic opioid administration (Duggan & North, 1983). Much of this controversy is avoided in the current investigation, which compares the effects of morphine on the amount of SP and CGRP released over fixed time periods.

There are also questions about studies demonstrating a decrease in opiate binding sites following degeneration of primary afferent fibres. Firstly, is trans-synaptic degeneration responsible for the disappearance of post-synaptic opiate binding sites? Secondly, in binding studies of synaptosomal homogenates, what is the relative contribution of presynaptic, postsynaptic and internalized receptors (inside-out synaptosomes) to the overall binding observed? Thirdly, are opioid peptides transported like oxytocin and vasopressin, and if so are the reports of sensory nerve transport of opiate receptors (Laduron, 1984a) detecting a neurophysin-like transport protein?

The fact that a proportion of opiate binding sites do remain supports the presence of opiate receptors on post-synaptic neurones that survive the degeneration of C fibres.

As discussed previously, there remains the possibility that morphine inhibits the release of another putative transmitter from nociceptive afferents, which is responsible for the analgesic effect in the spinal cord. Some C fibre-mediated flexor reflexes have been shown to be insensitive to high doses of morphine (5 mg/kg), suggesting that morphine sensitive and morphine insensitive transmitter systems may be a feature of C afferent fibres (Woolf & Wall, 1986b).

A different approach to this question of opiate effects on primary afferent terminals has demonstrated quite elegantly, that the actions of morphine are post-synaptic (Harris & Ryall, 1988). Since inhibitory responses of dorsal horn neurones must derive from primary afferent fibres as do the excitatory responses, a decrease in the release of transmitter by a presynaptic action of morphine should reduce inhibitory responses as well as excitatory responses. These workers showed a selective reduction by microelectrophoretic or systemic (0.5 - 6 mg/kg) morphine of only the excitatory and not the inhibitory responses evoked by a nociceptive stimulus, indicating that morphine acts after the first central synapse.

A small number of neurones which project from the substantia gelatinosa to deeper laminae (III - IV) neurones have been described (Light & Kavookjian, 1988), which lends anatomical support to these findings. These authors also suggested that the selective effects of morphine in the substantia gelatinosa and not the deeper laminae (Duggan *et al.* 1977a) may arise from a selective distribution of post-synaptic opiate receptors on the somata of these neurones.

Studies of enkephalin distribution in the superficial dorsal horn (Hunt *et al.* 1980; Glazer & Basbaum, 1983) have not supported the hypothesis of a

presynaptic control, by enkephalins, of transmitter release from the central terminals of nociceptors (Jessell & Iversen, 1977). It has been mentioned previously (pp. 70-71) that axoaxonic synpases are rare, if not absent, in the substantia gelatinosa, which does not support a presynaptic inhibition by opiates of the release of transmitter. It is possible that peptides could spread diffusely from sites of release (Iversen, 1986) and thus not require axoaxonic synapses to exert a presynaptic control of the terminals of nociceptors. In the vertebrate nervous system, however, such a proposal is still conjectural. In all, the present results support the contention that the analgesic effects of morphine administration systemically or directly to the spinal cord surface do not occur by a reduction in the release of SP or CGRP.

Morphine Dependence and Withdrawal

Introduction

Although the previous chapter has shown no effect of acute morphine on the release of sensory neuropeptides, the chronic administration of morphine may involve separate pharmacological mechanisms. For example, while the level of irSP in the rat dorsal horn was not affected by acute morphine administration, there were significantly elevated levels following chronic morphine, and the striatum and medulla showed larger increases in irSP (Bergström *et al.* 1984). The chronic administration of morphine has also been shown to enhance irSP immunostaining in rat dorsal horn (Vacca *et al.* 1980), and this was interpreted as an accumulation over time of SP in the terminals of SP-containing C fibres. In a neonatal spinal cord slice preparation exposed to morphine superfusion for 1 h, naloxone enhanced the ventral root depolarization induced by capsaicin (presumably due to an increased efflux of irSP), but produced no increased responsiveness to SP superfusion (Bell & Jaffe, 1986). Since a putative SP antagonist also partially blocked the ventral root depolarization produced by capsaicin, it was concluded that the morphine

withdrawal response in the spinal cord is mediated at a presynaptic site (Bell & Jaffe, 1986). Behavioural experiments in morphine-dependent mice have associated a naloxone-induced jumping behaviour with enhanced SP release (Ueda *et al.* 1987). The behaviour is blocked by a SP antagonist and naloxone was found to enhance radioactive calcium entry into spinal synaptosomes prepared from morphine-dependent mice, suggesting increased transmitter release would result (Ueda *et al.* 1987).

In morphine-dependent cats, the microelectrophoretic administration of naloxone into the substantia gelatinosa produces a greatly enhanced response of dorsal horn interneurones to impulses in nociceptive afferent fibres (Johnson & Duggan, 1981; Johnson & Duggan, 1984). It has been proposed that the increase in excitability of spinal neurones during morphine withdrawal is due to an increased efflux of SP from primary afferent fibres and/or a post-synaptic receptor supersensitivity to normal rates of transmitter release (Bergström *et al.* 1984).

The objectives of these studies,then, were to examine the non-noxious and noxious evoked release of irSP and irCGRP during morphine dependence and during the opiate withdrawal reaction precipitated by naloxone.

Results

Release of irSP during morphine withdrawal

It is evident from **Fig. 44** that neither non-noxious (**A**) nor noxious (**C**) stimulation of the ipsilateral afferent fibres by cutaneous or electrical modes evoked a significantly greater irSP release following morphine withdrawal brought on by naloxone (**Fig. 44 B,D**).

While this is the major comparison sought after, minor observations are that the basal release of SP by non-noxious stimulation appears to be larger than previously observed, and that the surface release of irSP is not as prominent in these series of experiments.

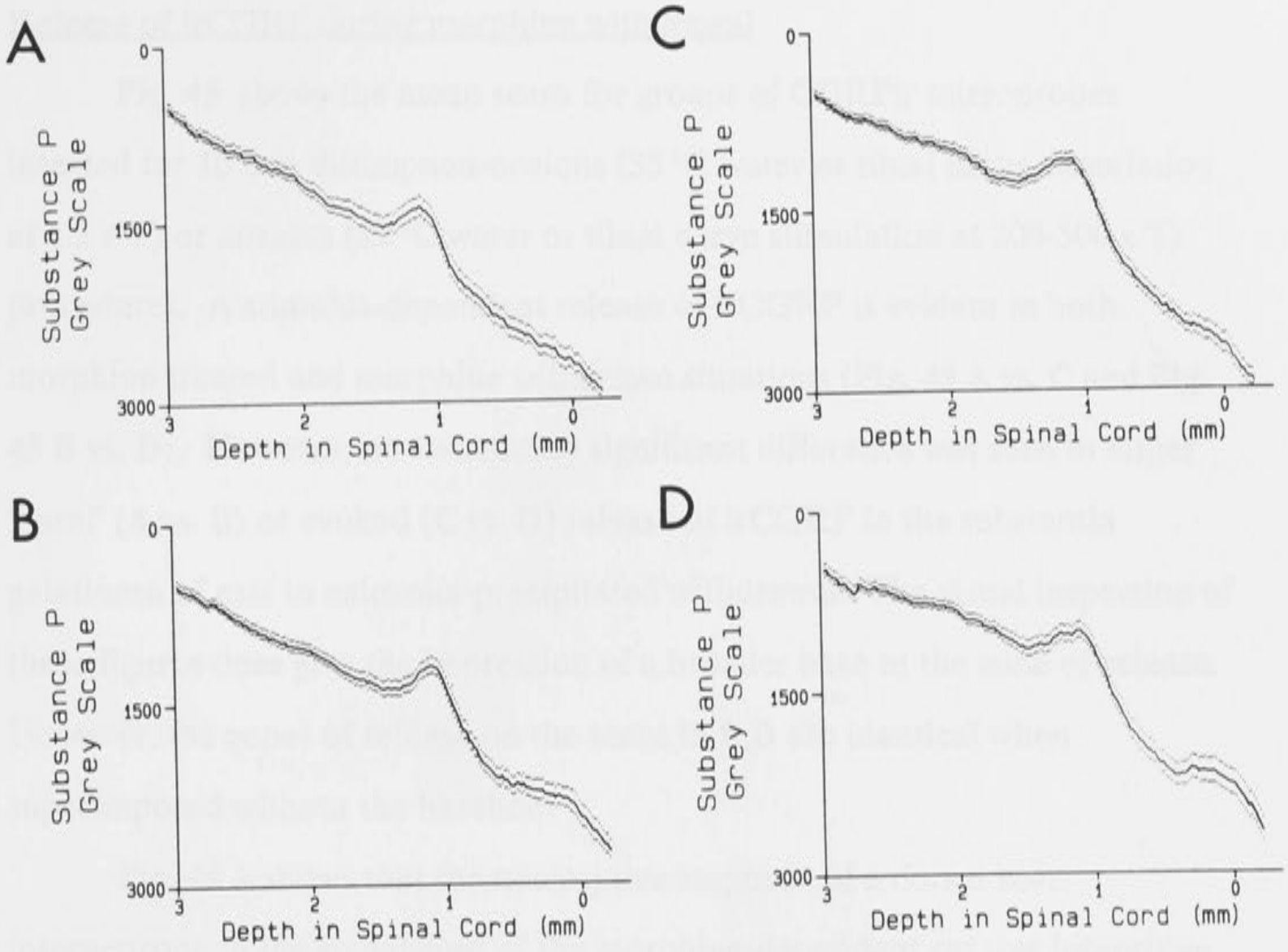


Fig. 44. Naloxone-precipitated morphine withdrawal is not associated with an increased efflux of irSP in the cat substantia gelatinosa.

A,B. Non-noxious heating (35^{O}C water) or no stimulation for 10 or 20 min ipsilateral to the placement of microprobes.

A. Pre-naloxone controls (N = 45).

B. Post-naloxone (N = 46).

C,D. Noxious heating (50^{O}C water) or electrical stimulation of the tibial nerve at C fibre threshold (200 - 300 x T) was applied for 10 or 20 min ipsilateral to the placement of microprobes.

C. Pre-naloxone controls (N = 49).

D. Post-naloxone (N = 46).

Release of irCGRP during morphine withdrawal

Fig. 45 shows the mean scans for groups of CGRPir microprobes inserted for 10 min during non-noxious (35 °C water or tibial nerve stimulation at 1.5 x T) or noxious (52 °C water or tibial nerve stimulation at 200-300 x T) procedures. A stimulus-dependent release of irCGRP is evident in both morphine treated and morphine withdrawn situations (**Fig. 45 A** vs. **C** and **Fig. 45 B** vs. **D**). However, no statistically significant difference was seen in either 'basal' (**A** vs. **B**) or evoked (**C** vs. **D**) release of irCGRP in the substantia gelatinosa of cats in naloxone-precipitated withdrawal. The visual inspection of these figures does give the impression of a broader base to the zone of release. However, the zones of release on the scans in **B,D** are identical when superimposed without the baseline.

Fig. 46 A shows that the nociceptive response of a dorsal horn interneurone in the spinal cord of the morphine-dependent cat was insensitive to an analgesic dose (1 mg/kg i.v.) of morphine but hypersensitive to the same nociceptive stimulus during the withdrawal reaction. It is probably not correct to assume that the animal was also tolerant, since this implies that the same endpoint of gated C fibre depression would have been obtained with higher morphine doses, and this was not tested here. However, the presence of withdrawal hyperexcitability in the spinal cord in these experiments do verify that the animals treated with this dose regime were dependent on morphine. **Fig. 46 B** is an example of the results obtained with antibody microprobes prepared to detect immunoreactivity of two sensory neuropeptides, SP (**A,B**) and CGRP (**C,D**), both showing similar zones of release in the substantia gelatinosa, before and after a naloxone-precipitated withdrawal in the morphine dependent cat.

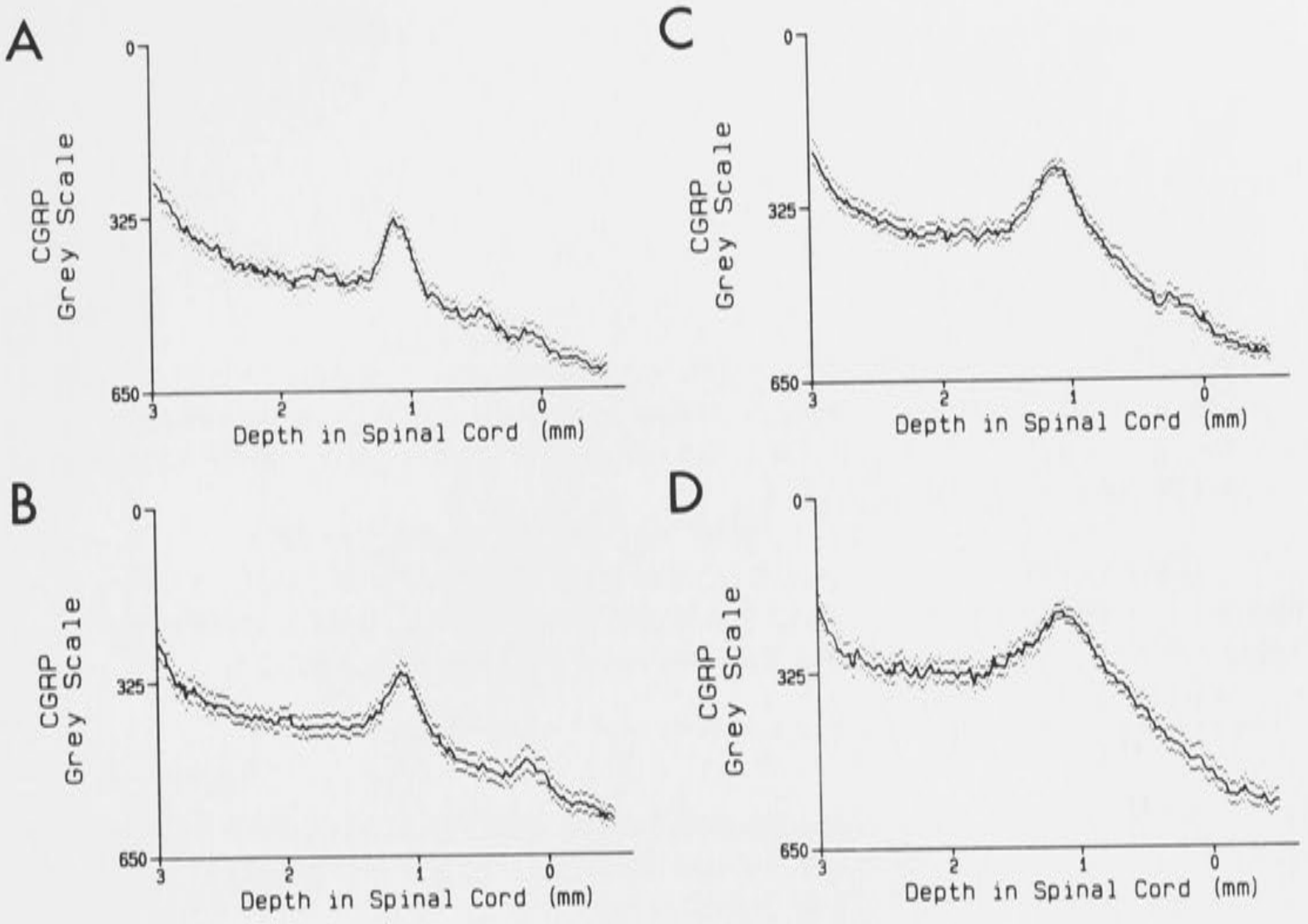


Fig. 45. Naloxone-precipitated morphine withdrawal is not associated with an increased efflux of irCGRP in the cat substantia gelatinosa.

A,B. Non-noxious heating (35^{o}C water) or electrical stimulation of the tibial nerve at A fibre threshold (1.5 x T) was applied for 10 min ipsilateral to the placement of microprobes.

A. Pre-naloxone controls (N = 29).
B. Post-naloxone (N = 32).

C,D. Noxious heating (52^{o}C water) or electrical stimulation of the tibial nerve at C fibre threshold (200 - 300 x T) was applied for 10 min ipsilateral to the placement of microprobes.

C. Pre-naloxone controls (N = 33).
D. Post-naloxone (N = 36).

Fig. 46. Naloxone-precipitated morphine withdrawal is not associated with an increased efflux of sensory neuropeptides in the cat substantia gelatinosa. The analgesic action of morphine does not result from a presynaptic inhibition of release of sensory neuropeptides.

A. Gated C-fibre response of a dorsal horn interneurone (depth 1.62 mm) excited by electrical stimulation of the tibial nerve at 50 V, 0.5 ms pulse width, repeated at 0.3 Hz in a cat chronically treated with morphine for 3 d.

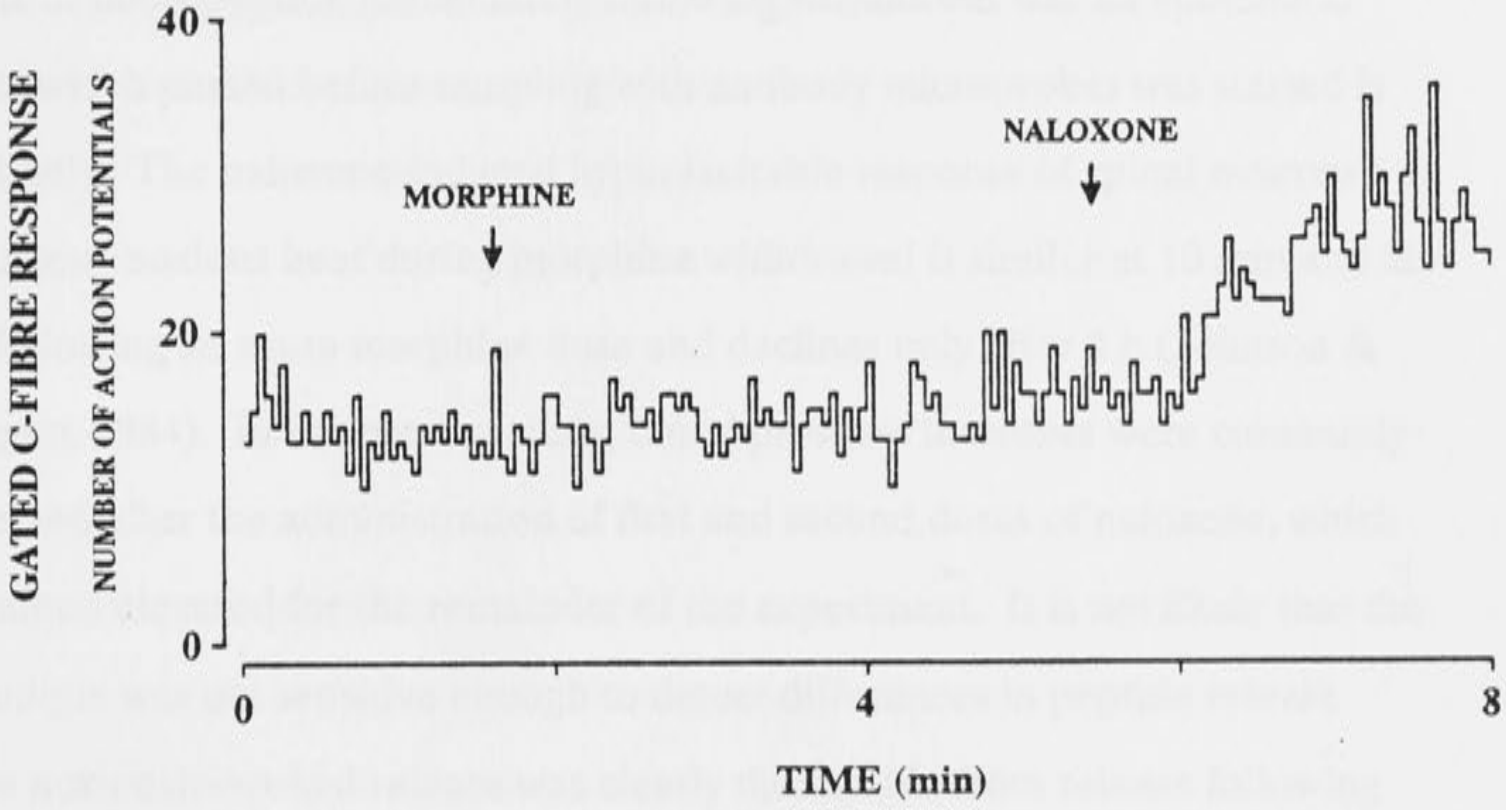
Morphine i.v. 1 mg/kg
Naloxone i.v. 0.5 mg/kg

B. Photographic enlargements of autoradiographic microprobe images showing zones of neuropeptide release arranged on a line drawing of a transverse section of the lumbar spinal cord.

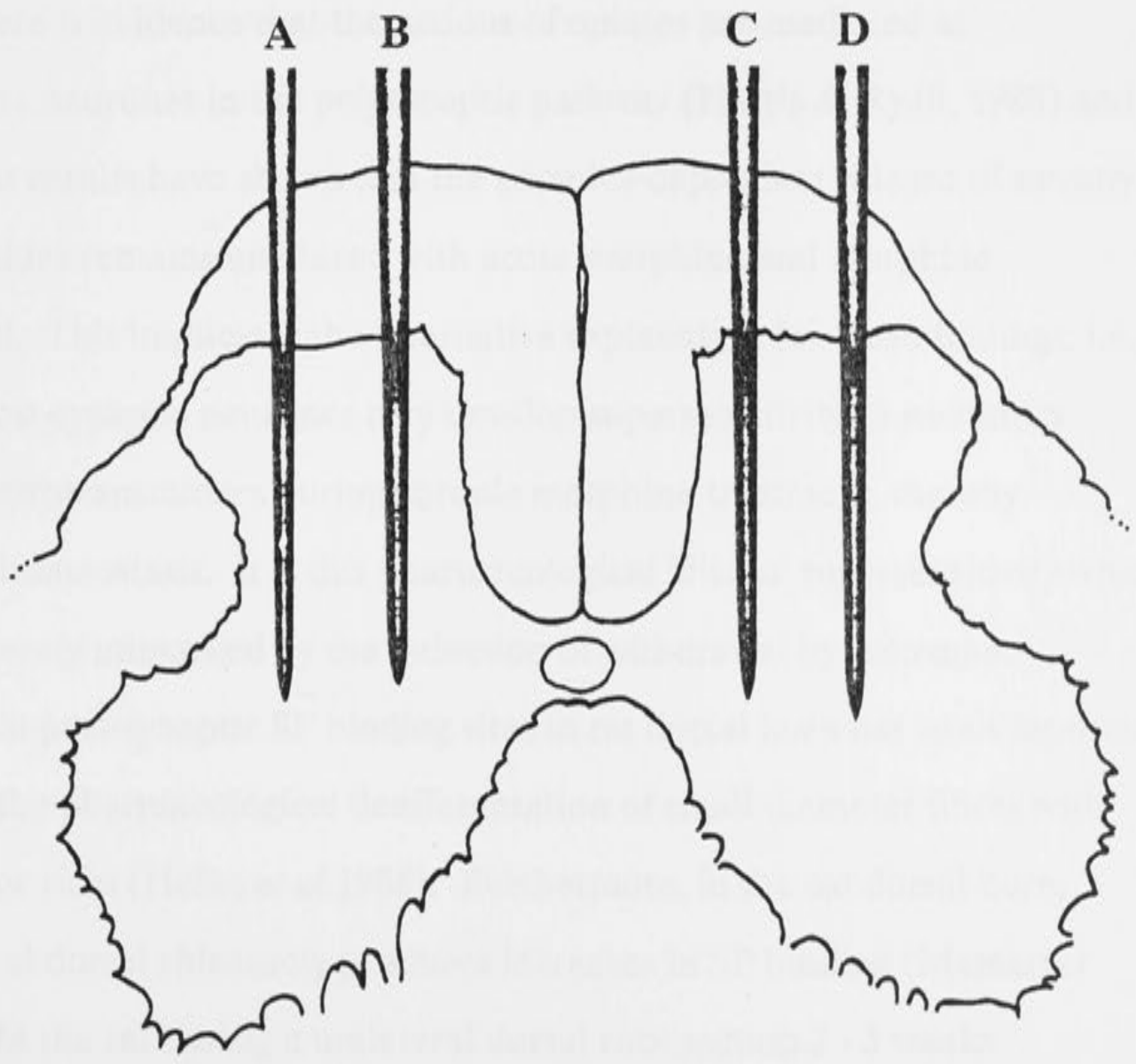
A,B: IrSP, Probe stimuli were 50°C water for 15 min.
A. Pre-naloxone.
B. Post-naloxone.

C,D: IrCGRP, Probe stimuli were 52°C water for 10 min.
C. Pre-naloxone.
D. Post-naloxone.

A


GATED C-FIBRE RESPONSE
NUMBER OF ACTION POTENTIALS
40
20
0
MORPHINE
NALOXONE
0
4
8
TIME (min)

B


A
B
C
D

Discussion

Many of the arguments that were put forth in the previous chapter on effects of acute morphine are also applicable to the discussion of the present findings with chronic morphine treatment. The possibility that an exaggerated efflux of neuropeptide immediately following withdrawal was an ephemeral event which passed before sampling with antibody microprobes was started is not likely. The naloxone-induced hyperexcitable response of spinal neurones to cutaneous noxious heat during morphine withdrawal is similar at 10 min and at 4 h following an acute morphine dose and declines only after 6 h (Johnson & Duggan, 1984). In the present study, blood pressure increases were commonly observed after the administration of first and second doses of naloxone, which remained elevated for the remainder of the experiment. It is not likely that the technique was not sensitive enough to detect differences in peptide release since noxiously-evoked release was clearly discernible from release following non-noxious stimuli.

There is evidence that the actions of opiates are mediated at penultimate neurones in the polysynaptic pathway (Harris & Ryall, 1988) and the present results have shown that the stimulus-dependent release of sensory neuropeptides remains unaltered with acute morphine and morphine withdrawal. This implicates the alternative explanation for these findings, i.e., that the post-synaptic neurones may develop supersensitivity to excitatory sensory neurotransmitters during chronic morphine treatment, thereby achieving homeostasis. It is this pharmacological 'disuse' supersensitivity which is subsequently unmasked by the induction of withdrawal by naloxone. Increases in post-synaptic SP binding sites in rat dorsal horn has been reported following the pharmacological deafferentation of small diameter fibres with capsaicin or ricin (Helke *et al.* 1986). Furthermore, in the cat dorsal horn, lumbosacral dorsal rhizotomy produces increases in SP binding (Massari *et al.* 1985). In the rat having a unilateral dorsal root section 2 - 3 weeks

previously, the electrophoretic application of the SP homologue, eledoisin related peptide, produced greater excitation from dorsal horn neurones on the deafferented side than on the intact side (Wright & Roberts, 1978), suggesting a denervation supersensitivity of tachykinin receptors. Whether or not the chronic treatment with morphine induces an exaggerated responsiveness to electrophoretic SP remains to be determined.

Effects of Baclofen on Release of Substance P

Introduction

Baclofen, (*p*-chlorophenyl GABA) (Pierau *et al.* 1975) is used clinically as an antispastic agent (Knutsson *et al.* 1974) but has also been reported to be analgesic following intrathecal administration in humans. In experimental animals i.v., i.p., or i.t. (-)-baclofen has been shown to reduce nociceptive sensation in several tests of analgesia (Wilson & Yaksh, 1978; Panerai *et al.* 1985; Vaught *et al.* 1985; Sawynok & Dickson, 1985).

A presynaptic action of baclofen at primary afferent terminals which inhibits release of excitatory transmitter in the absence of terminal depolarization, appears to underlie the reduction of primary afferent excitatory transmission in the spinal cord (Pierau & Zimmermann, 1973; Curtis *et al.* 1981; Dickenson *et al.* 1985). In the cat, the systemic administration of (±)-baclofen reduces mono- and polysynaptic EPSP's in motoneurones but produces neither hyperpolarisation nor changes in resting membrane conductance (Pierau & Zimmermann, 1973). However, all synapses would be exposed to baclofen following systemic administration and the relative sensitivity of polysynaptic vs. monosynaptic pathways is not possible to assess. The microelectrophoretic administration of (-)-baclofen in the cat spinal cord has demonstrated that monosynaptic excitation of spinal neurones by impulses in primary afferent fibres is more sensitive than descending excitatory or polysynaptic pathways (Curtis *et al.* 1981). Most of these studies have examined large diameter fibres

but baclofen is also effective in reducing the excitation of dorsal horn neurones by impulses in high (120T) threshold primary afferent fibres of the tibial nerve (Curtis *et al.* 1981). In rats, both intrathecal and intravenous baclofen (1 - 3 mg/kg) reduced C fibre -evoked responses in dorsal horn neurones in a stereospecific and bicuculline-insensitive manner. In decerebrate cats, (±)-baclofen (1 mg/kg) blocked the asynchronous late response in spinal interneurones activated by high intensity transcutaneous electrical stimulation of C fibres (Piercey & Hollister, 1979). Surprisingly, the A fibre component was little affected, leading the authors to conclude that baclofen exerted its effects mainly on spinal interneurones when administered systemically.

While others have suggested that supraspinal sites may be involved in the antinociceptive action of low doses of baclofen (Sawynok, 1983; Sawynok & Dickson, 1985), quite potent effects are seen in spinal preparations *in vivo* and in spinal slices *in vitro* suggesting a direct spinal action. In the isolated spinal cord of immature rats, a contralateral slow ventral root potential produced by a 10 V stimulus to the dorsal root was reduced by 0.01 to 0.1 μM (±)-baclofen applied to the bath (Akagi & Yanagisawa, 1987). The monosynaptic reflex was inhibited at a dose of baclofen 10 - fold greater (1.0 μM) than that required to reduce the slow reflex. Muscimol and diazepam also inhibited the reflex and all three drugs were not antagonized by bicuculline. Furthermore, the slow ventral root potential was mimicked by SP which showed a similar pharmacological profile of sensitivity to muscimol, diazepam and bicuculline (Akagi & Yanagisawa, 1987), suggesting that SP is the transmitter released by C fibres to produce these post-synaptic effects.

This hypothesis would be supported by observing a reduction in the release of SP coupled with a decrease in the C fibre response. Few reports have examined the effects of baclofen on neuropeptide release from the CNS *in vivo*, therefore the purpose of this study was to examine the possible presynaptic action of systemic baclofen on the release of irSP in the substantia gelatinosa.

Results

The total number of probes for these experiments was 123, of which 67 comprised the group for comparison of A + C fibre stimulation of the tibial nerve for 15 min before and after baclofen administration, and 56 comprised the group of noxious cutaneous stimulation inclusive of 50 °C heat and noxious mechanical stimulation for 20 min both pre- and post-baclofen.

As shown in previous chapters, stimulation of the tibial nerve at an intensity sufficient to excite C fibres produced a release of irSP in the substantia gelatinosa of the spinal cord (**Fig. 47 A**). It is apparent from **Fig. 47 B** that there was no effect of systemic administration of 4 mg/kg (±)-baclofen on the height or shape of the zone of release of SP. The zone at the cord surface was present on the mean scan of the post-baclofen group, likely due to the fact that these probes were inserted in the second half of the experiment. A similar result was found with irSP release evoked by noxious mechanical and thermal stimuli to the ipsilateral hindpaw (**Fig. 47 C,D**). No significant reduction in SP release was seen at the level of the substantia gelatinosa, even with the highest dose of 10 mg/kg of (±)-baclofen administered i.v.

To confirm that the doses employed were effective in reducing polysynaptic excitation of dorsal horn interneurones by high threshold tibial afferent fibres, single unit recording was carried out on single neurones by means of a 4 M NaCl-filled microelectrode (10 - 20 Mohms), with techniques described in the Methods section. Systemic (±)-baclofen decreased the gated C fibre response to high intensity stimulation of the tibial nerve, an effect which was rapid in onset (< 1 min) and continued to a stable minimum value by 7 min (**Fig. 48**). Recovery was not demonstrated here due to the slow time course of return of responsiveness (Curtis *et al.* 1981). The histograms of **Fig. 49** show the latency record of excitatory responses to high intensity tibial nerve stimulation, both pre- and post-baclofen at the times indicated in **Fig. 48**. The short and

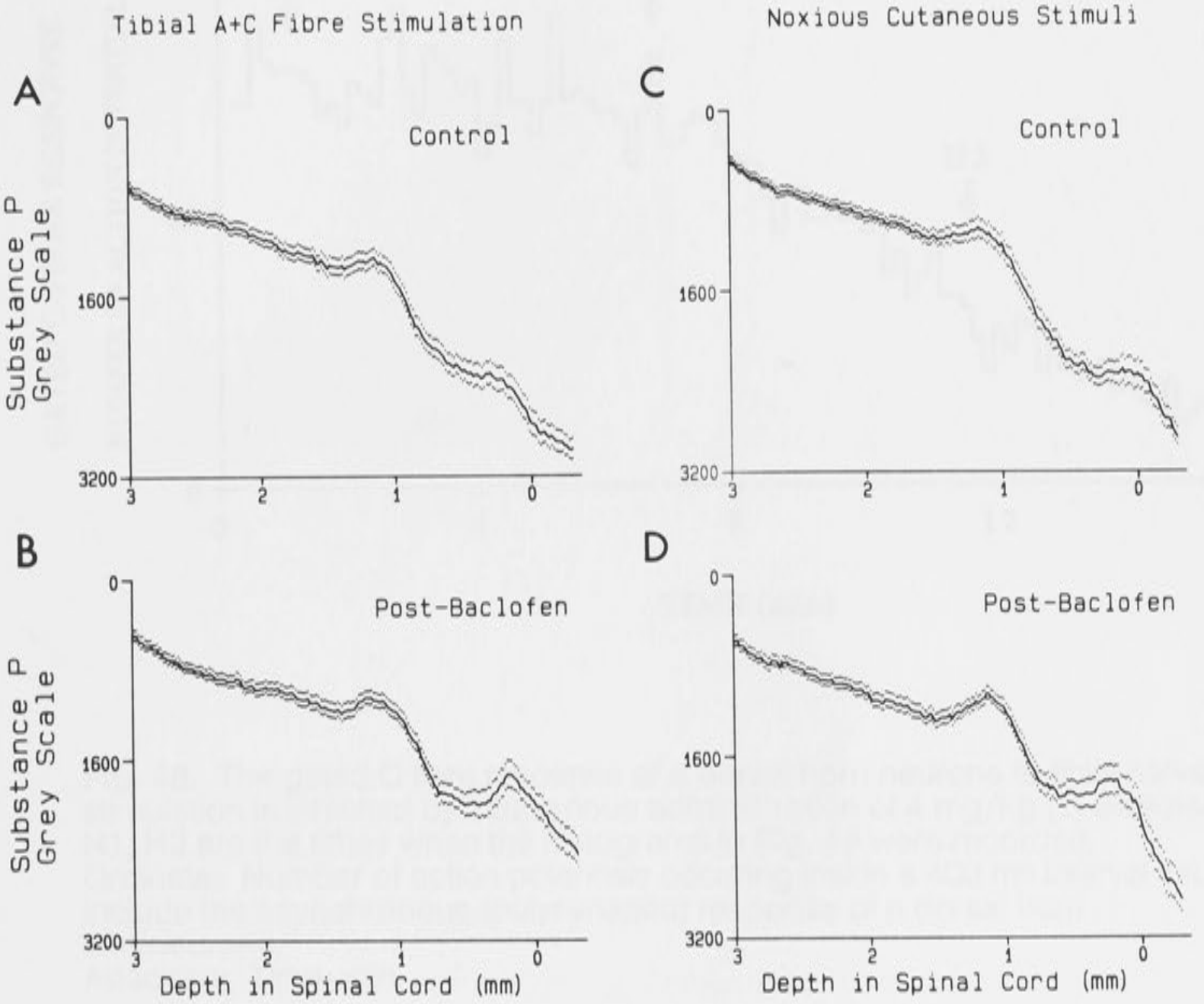


Fig. 47. Lack of effect of (±)-baclofen on the release of substance P in the substantia gelatinosa.

A,B Release of irSP evoked by electrical stimulation of the tibial nerve at an intensity sufficient to excite C fibres before (A) and after (B) the intravenous administration of 4 mg/kg (±)-baclofen. The mean scan in A and B was calculated from 34 and 33 individual microprobes, respectively.

C,D Noxious mechanical stimulation and noxious heating of the hind limb were used to evoke irSP release. The mean scan lines are of 29 and 27 microprobes inserted before (C), and after (D) the administration of 10 mg/kg (±)-baclofen, respectively.

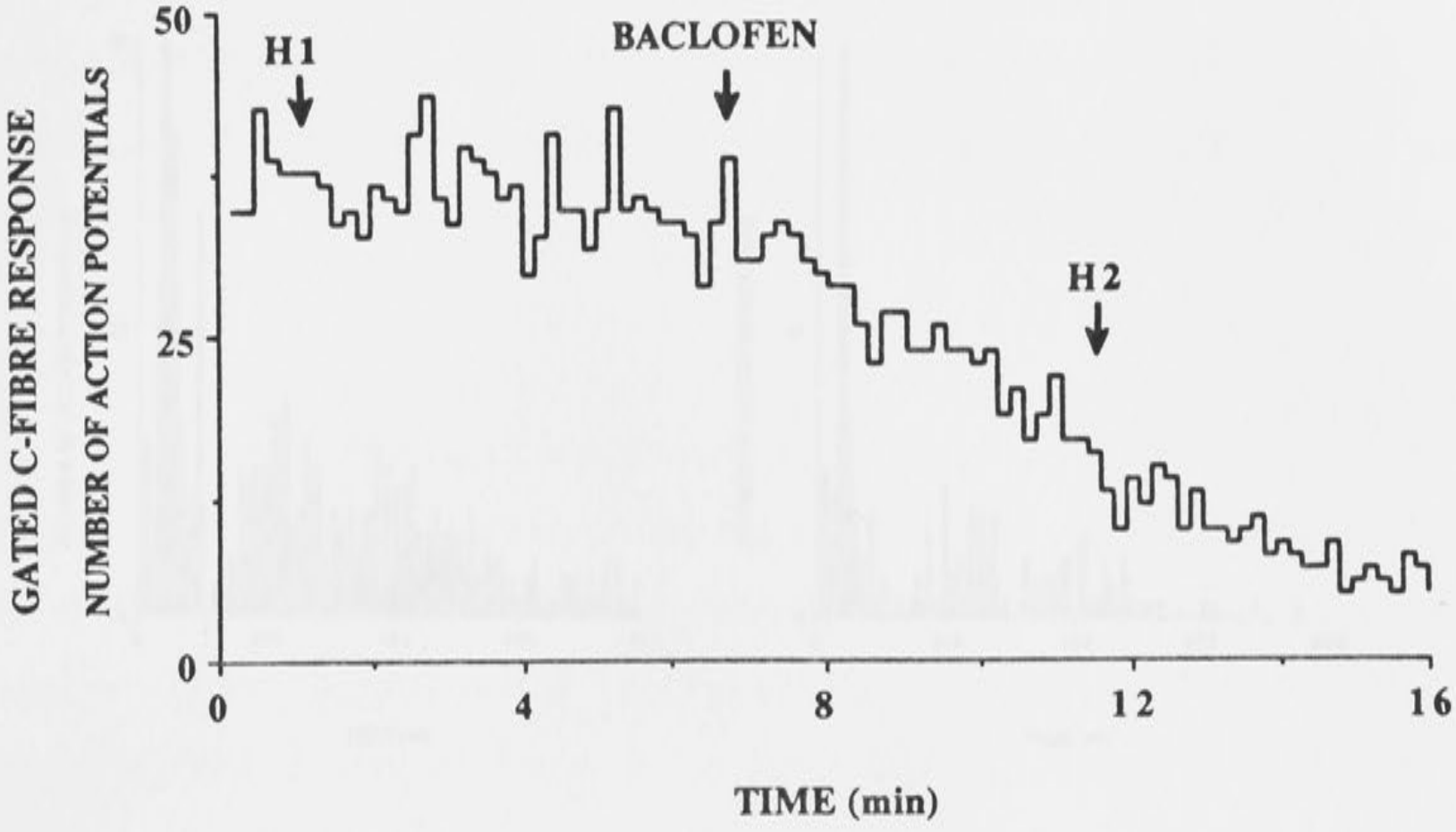


Fig. 48. The gated C fibre response of a dorsal horn neurone to tibial nerve stimulation is inhibited by intravenous administration of 4 mg/kg (±)-baclofen. H1, H2 are the times when the histograms in **Fig. 49** were recorded.
Ordinate: Number of action potentials occuring inside a 400 ms interval set to include the asynchronous (polysynaptic) response of a dorsal horn interneurone.
Abscissa: Time, min.

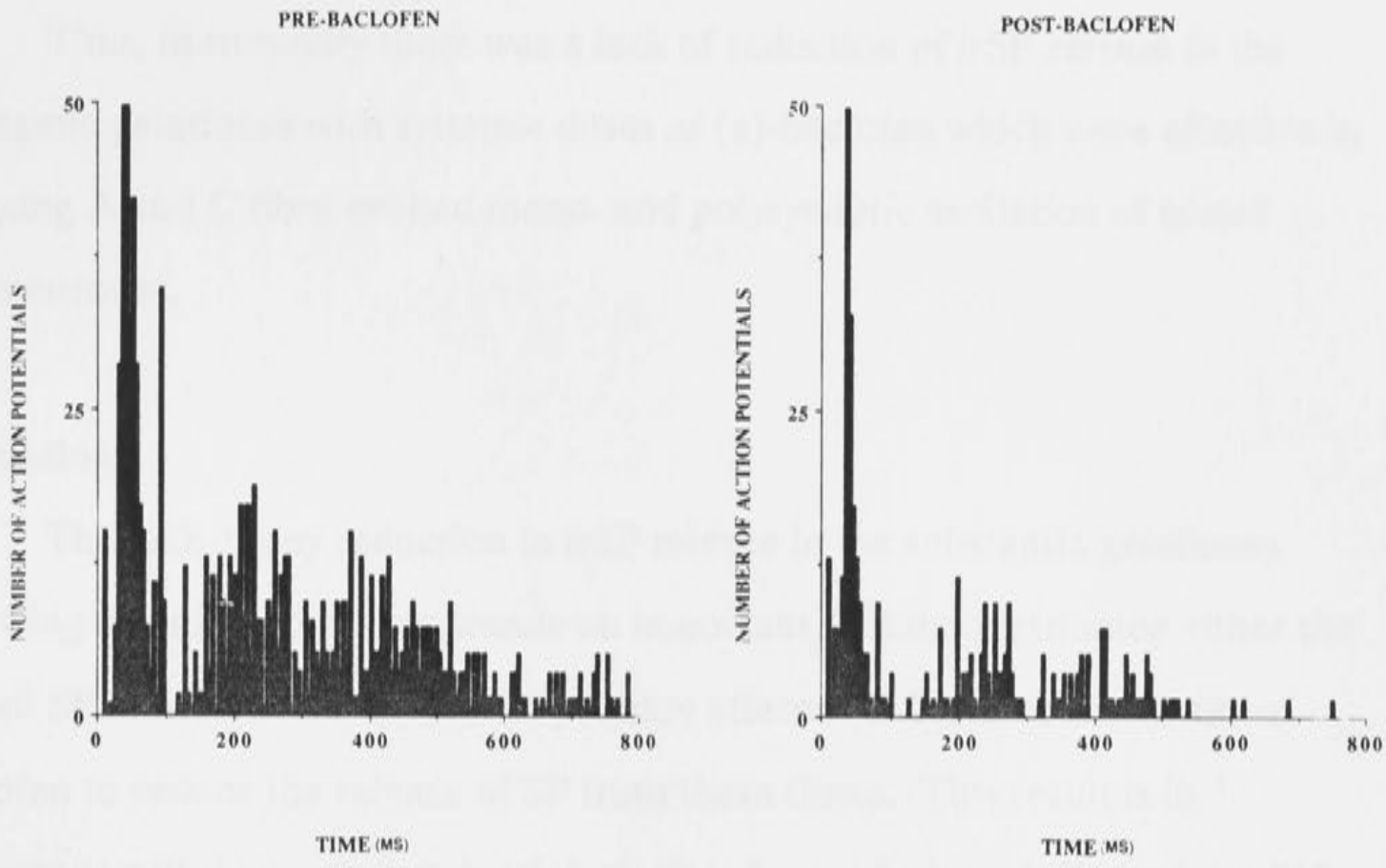


Fig. 49. Histograms of the latency of excitation of a dorsal horn interneurone by impulses in A and C afferents of the tibial nerve. The stimulus intensity was 200 - 300 times the threshold for the most excitable fibres, with a 0.5 ms pulse width.

A: Control histogram recorded pre-baclofen, at time indicated by H1 in **Fig. 48**.

B: Same cell recorded 6 min after 4 mg/kg (±)-baclofen, i.v., at time indicated by H2 in **Fig. 48**.

Ordinate: Number of action potentials in consecutive 0.5 ms bins (16 sweeeps) at 0.1 Hz. Abscissa: Time, ms.

long latency responses were decreased with apparently equal proportions. In the histogram taken at the time of maximal effect of baclofen where there were some short latency fibres still active but few asynchronous late responses (not shown).

Thus, in summary there was a lack of reduction of irSP release in the substantia gelatinosa with systemic doses of (±)-baclofen which were effective in reducing A and C fibre evoked mono- and polysynaptic excitation of spinal interneurones.

Discussion

The lack of any reduction in irSP release in the substantia gelatinosa following baclofen administration is an important finding questioning either the role of SP as a neurotransmitter of primary afferent C fibres or the ability of baclofen to reduce the release of SP from these fibres. This result is in agreement with an *in vitro* study of the isolated rat spinal cord slice, where 250 μM (-)-baclofen or 500 μM (±)-baclofen failed to reduce SP release evoked by (calcium-dependent) potassium stimulation (Sawynok *et al.* 1982).

<u>Scenario 1 - Baclofen inhibits release of a nociceptive transmitter unrelated to substance P</u>

It is possible that the sub-population of SP-containing neurones are not sensitive to the effects of baclofen. As reported in the section on SP localisation, SP is present in some, but not all (about 20%) small diameter DRG neurones. It is not likely that the technique used in this study was not sensitive enough to detect a reduction in SP release since the majority (about 70%) of SP in the substantia gelatinosa derives from primary afferent fibres. Even if these neurones are sensitive to baclofen, the reduction in nociceptive responsiveness may be due to another putative transmitters colocalized in the same neurone. For example, ir-glutamate has been found in small clear vesicles

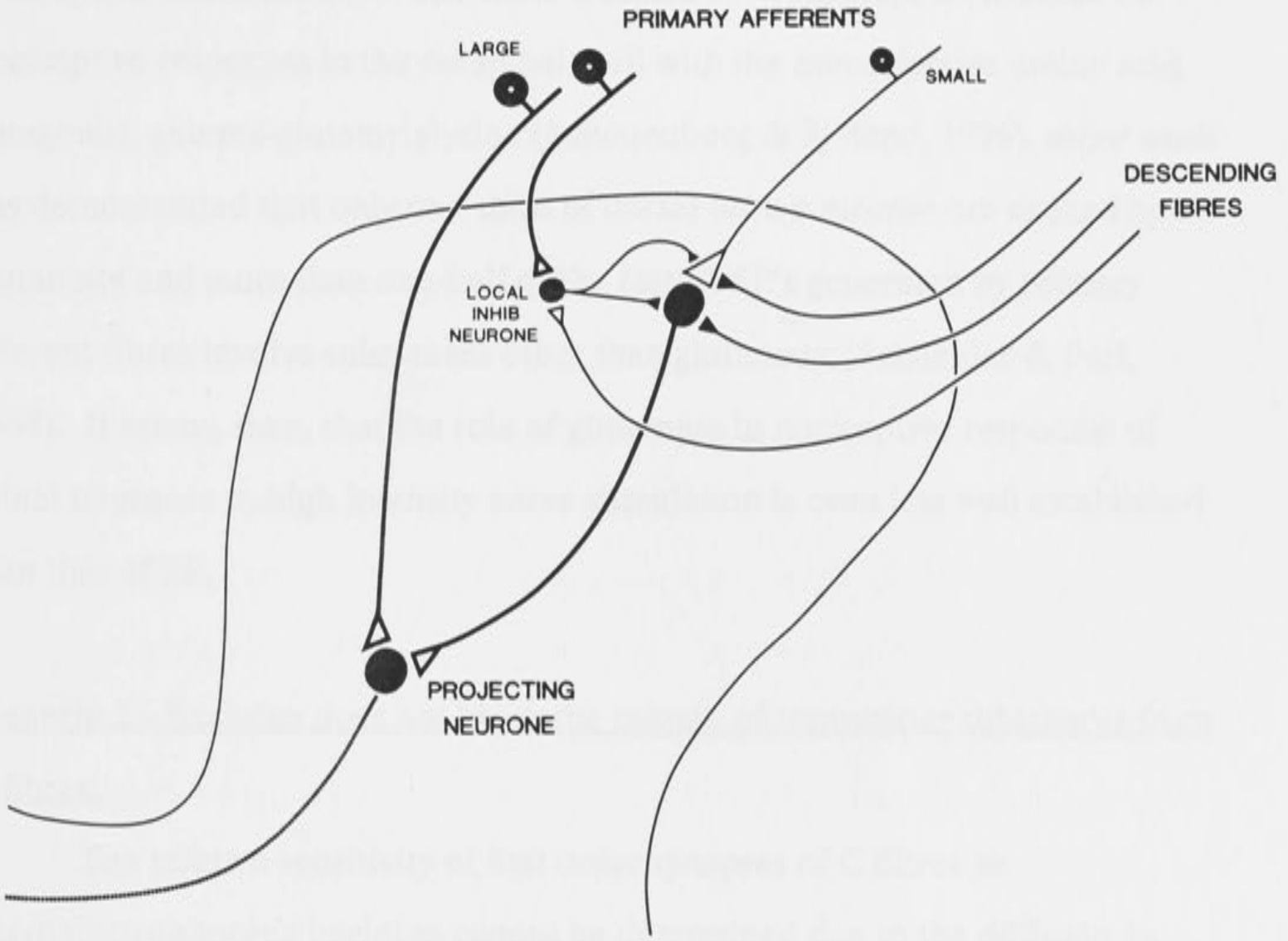


Fig. 50. Diagram of the neuronal pathways in the dorsal horn. Open trangles represent excitatory synapses and solid triangles represent inhibitory synapses.

in the same nerve terminals and a selective inhibition of the release of glutamate (but not SP) may be produced by baclofen. Although such a mechanism has not been demonstrated, it has been suggested that a selective distribution of phosphoproteins on the outside of synaptic vesicles may regulate the release of particular vesicular populations (Steiner *et al.* 1986; Walaas *et al.* 1986). This interpretation attributes an important role for glutamate in nociceptive transmission. While some workers have reported a blockade of nociceptive responses in the rat spinal cord with the non-selective amino acid antagonist, gamma-glutamylglycine (Schouenborg & Sjölund, 1986), other work has demonstrated that only one-third of dorsal horn neurones are excited by glutamate and more than one-half of the fast EPSP's generated by primary afferent fibres involve substances other than glutamate (Schneider & Perl, 1988). It seems, then, that the role of glutamate in nociceptive responses of spinal neurones to high intensity nerve stimulation is even less well established than that of SP.

Scenario 2 - Baclofen does not block the release of transmitter substances from C fibres.

The relative sensitivity of first order synapses of C fibres to microelectrophoretic baclofen cannot be determined due to the difficulty in identifying these monosynaptic excitations in the asynchronous input following a high intensity nerve stimulation. In the absence of this information, indirect evidence for presynaptic mechanisms of C fibres must be considered.

The apparent lack of axoaxonic synaptic arrangements involving C fibres has been discussed previously and does not support the notion of presynaptic inhibition of C fibres. A recent ultrastructural study has confirmed these findings for irSP profiles in the rat substantia gelatinosa (Ribeiro-Da-Silva *et al.* 1989). Terminals of irSP primary afferent fibres in lamina I are simple, and in ventral lamina II are more complex, having a glomerular structure with many

peripheral profiles. In agreement with studies discussed previously, vesicle-containing profiles are rarely presynaptic to irSP profiles. In contrast, the non-SPir profiles in the substantia gelatinosa are more complex and are surrounded by profiles which may be GABAergic axoaxonic synapses. This is agreement with earlier non-quantitative studies of irGAD profiles surrounding HRP labeled afferent fibres of undefined type (Barber *et al.* 1978b).

Furthermore, neither segmental inhibition from activity in low threshold afferents nor blockade of descending inhibition was found to inhibit presynaptically SP release under the conditions of the present experiments. In a similar experimental preparation, baclofen does not inhibit the release of transmitter from excitatory interneurones nor from descending fibres in the dorsolateral funiculus (Curtis *et al.* 1981). Physiological, pharmacological and anatomical evidence is consistent with the suggestion that the release of the C fibre transmitter is not affected by baclofen.

In contrast, there is evidence that baclofen mediates post-synaptic inhibition of deep neurones of the immature rat spinal cord (Allerton *et al.* 1989). At a concentration effective in reducing polysynaptic EPSP's, baclofen produced hyperpolarizing responses associated with an increased postsynaptic membrane conductance of potassium, but the decrease in membrane potential was quite small. After a systemic dose of baclofen, a small postsynaptic effect on large numbers of synapses throughout the spinal cord may outweigh the effect on one type of first order synapse.

Indirect pharmacological evidence also appears contrary to a presynaptic action of baclofen on SP-containing C fibres. The selective action of capsaicin on C fibres but not A fibres of the vagus nerve may be due to specific calcium channels located on these fibres (Marsh *et al.* 1987). Since the inhibitory action of baclofen on rat DRG neurones in primary culture is also thought to be mediated through calcium channels (Dolphin & Scott, 1987; Green & Cottrell, 1988), it may be that the action of baclofen is selective for only A afferent

fibres. This study gave no evidence for the types of cells examined and only the large cells in culture may have been selected simply due to ease of electrode penetration.

In summary, then, there is no evidence to support the notion that baclofen is capable of presynatically inhibiting release of transmitter from C fibres, and the observation of a lack of effect on SP release of systmeic baclofen cannot be used to rule out a role of SP in transmission of nociceptive information in the spinal cord.

7. CONCLUDING REMARKS

The criteria for the establishment of a neurotransmitter candidate in the peripheral nervous sytem have been successfully applied to the central nervous system in the past, and this success has also largely depended upon technological advances and an interdisciplinary approach to the question at hand. The antibody microprobe technique is capable of assessing the dynamic status of a particular peptidergic pathway in the central nervous system, and for this reason will continue to be a useful tool for neuropeptide research.

The fact that many neuropeptides are released in the substantia gelatinosa with the same stimulus, and that the same stimulus is capable of releasing many neuropeptides is an interesting facet of the neuropeptide concept. How does this relate to the conceptual development of theories of sensory physiology? The specificity theory of von Frey proposed almost a century ago, that the quality of each sensation arises from a few specialized nerve fibres responding to a specific sensory stimulus. In the 1950's Sinclair proposed the pattern theory which stated that it was the pattern of activity in many nerve fibres which results in the vast array of sensory submodalities. The modified pattern theory of Melzack & Wall (1962) was a consolidation of these theories with special consideration given to electrophysiological evidence of stimulus specificity of certain fibre types. The results of this thesis have shown a

specificity of somatostatin-containing neurones to thermal and not mechanical stimuli, but SP and CGRP are released with both forms of stimulation. Perhaps this is a hint that the specificity of neurones responding to specific stimuli may actually lie in the pattern of their chemical content. If this is borne out then perhaps von Frey was quite correct but only inaccurate in proposing that wherein lies the specificity is morphology and not the chemical code.

One should be aware that afferent input to the spinal cord activates many different systems and not all are immediately involved in sensory discrimination. Therefore, it would be incorrect to conclude that a particular neuropeptide is involved in the capacity of nociceptive transmission merely because its release is associated with nociceptive stimuli - the problem of cause and effect. Many peptides may fall in the category of 'necessary, but not sufficient' for a nociceptive response. Although this argument has been put forth for SP in nociceptive afferents (Wall & Fitzgerald, 1982), it may be even more applicable to CGRP which seems to exhibit very modest membrane effects on dorsal horn neurones. Kruger (1987, 1988) has discussed many interesting possibilities for the *raison d'être* of CGRP in fine sensory neurons including functions relating to efferent and afferent properties and events related to recovery and repair. It is an interesting possibility that individual peptides may have multifunctional roles in the spinal cord.

The data showing a lack of segmental and descending control of irSP release provide a consistent argument that the sensory signal conveyed by this neuropeptide is not 'edited out' by physiological control mechanisms on the first-order cells. This may reflect the potential importance of this information to the second-order cells, and phylogenetically conserved neural mechanisms which signal situations threatening existence of the organism.

The failure of morphine and baclofen to inhibit the release of some of these neuropeptides is also consistent with the above-mentioned data and emphasizes the robust nature of peptidergic communication. It also

underscores the importance of knowing the site of action of pharmacological agents, for if these drugs do act in part by a presynaptic mode then another canonical transmitter may be mediating the observed analgesic effects. These data may then provide evidence for a selective control of release of the contents of peptidergic and non-peptidergic vesicles within single neurons. If this mechanism is not operative, these data implicate post-synaptic sites of action of these drugs.

Far from the synapse being a simple relay, there is the potential for a wealth of information to be encoded in the chemical composition of peptidergic primary afferent neurones. Or, if I may borrow a saying of Prof. Duggan's, *"If we had all the answers, we wouldn't be here".*

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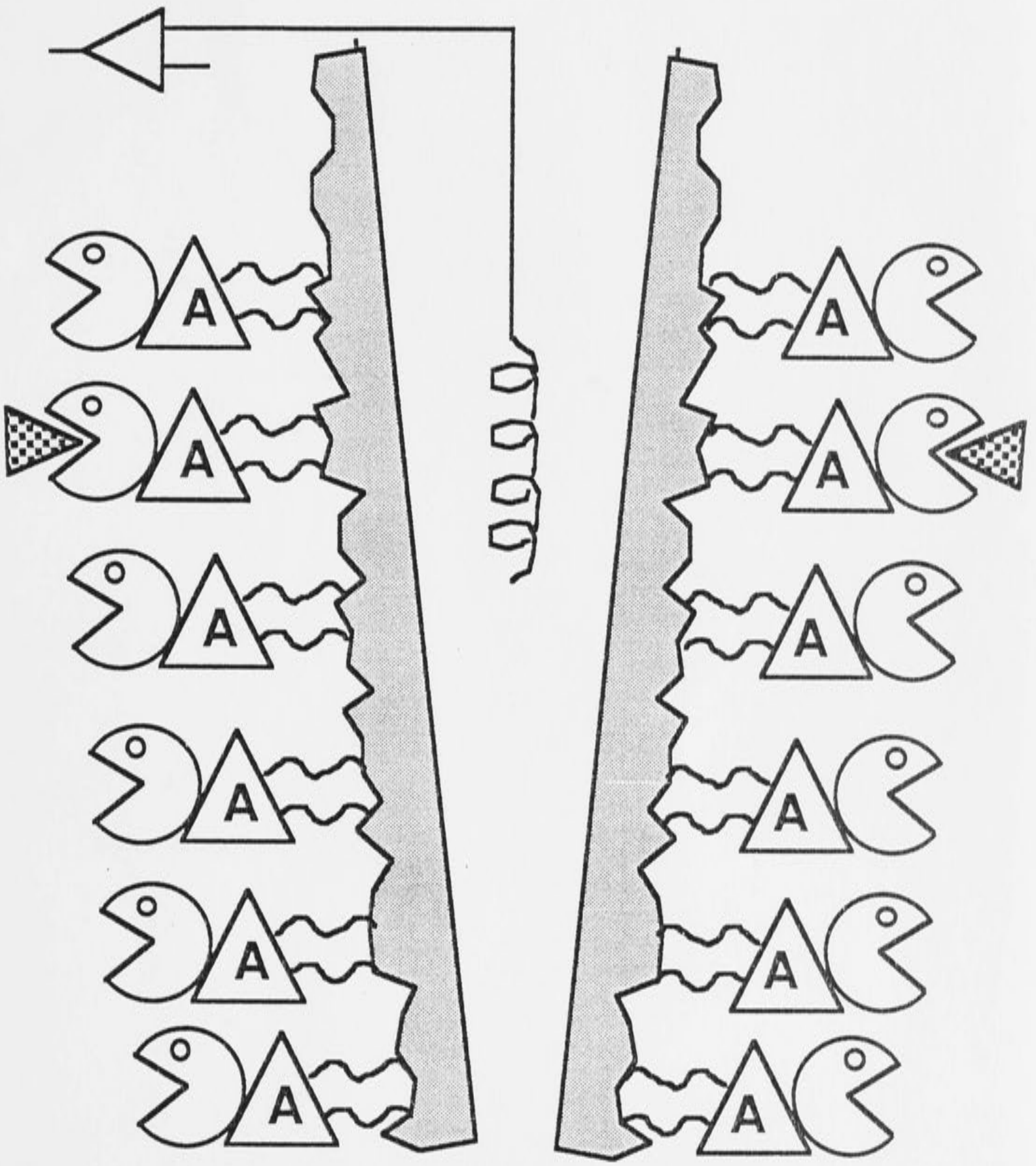
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