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Pharmacology of neuronal elements in the cat spinal dorsal horn

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PHARMACOLOGY OF NEURONAL ELEMENTS IN THE CAT SPINAL DORSAL HORN

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Pharmacology of neuronal elements in the cat spinal dorsal horn

by

Srdija Jeftinija

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LIST OF ABBREVIATIONS

CCK	Cholecystokinin
CNS	Central nervous system
p-CPA	p-Chlorophenylalanine
GABA	Gamma-aminobutyric acid
GDEE	L-Glutamic acid diethyl ester
GHRIF	Growth hormone release inhibiting factor (or somatostatin)
HA-966	1-Hydroxy-3-aminopyrolidone-2
5-HT	5-Hydroxytryptamine (serotonin)
i.p.	intraperitoneal
i.v.	intravenous
LTM	Low threshold mechanoreceptor
NE	Norepinephrine
NRM	Nucleus raphe magnus
NS	Nociceptive specific
NT	Neurotensin
PAD	Primary afferent depolarization
PAH	Primary afferent hyperpolarization
PNS	Peripheral nervous system
SP	Substance P
SPLI	Substance P-like immunoreactivity
SS	Somatostatin (or GHRIF)

TTX	tetrodotoxin
VIP	Vasoactive intestinal polypeptide
WDR	Wide-dynamic-range

INTRODUCTION

Explanation of Dissertation Format

This dissertation is written in the alternative format permitted by the Graduate College. It includes an objective, a literature review, a rationale, an experimental part, a brief discussion, a summary and a list of literature cited.

The experimental part is divided into six sections. Two of these sections, I and IV, correspond to research papers already published, sections II and V correspond to research papers currently in press, and sections III and VI contain data intended to be published.

This dissertation presents the results from a series of experiments conducted over three years by the author under the supervision of his major professor, Dr. Mirjana Randić. The results obtained from rat spinal cord slice preparation included in sections I and II were obtained by Dr. V. Miletić, Mr. K. Murase and Dr. V. Nedeljkov to whom all credits belong.

Objective

Anatomical and physiological studies have provided valuable information about the morphology and termination patterns of functionally identified primary afferent fibers and the location of neurons in the spinal dorsal horn. Immunocytochemical, fluorescence and a number of biochemical techniques have demonstrated that high concentrations of cholecystokinin (CCK), somatostatin (SS), substance P (SP), vasoactive intestinal polypeptide (VIP), L-glutamate, 5-hydroxytryptamine (5-HT) and norepinephrine (NE) are present in the superficial parts of the spinal dorsal horn, the

same area where primary afferent fibers of fine diameter (Aδ and C) are known to terminate. However, the identity of the neuronal elements responsive to some of these agents and the type of cellular response(s) in the spinal dorsal horn are not as yet clear. Therefore, the purpose of this work was to examine the actions of these substances on the excitability of functionally identified dorsal horn neurons and single cutaneous primary afferent fibers in the cat spinal cord in vivo.

This work was done on functionally identified spinal dorsal horn neurons and single primary afferent fibers in anesthetized or spinalized cats. Neuronal activity was monitored extracellularly while CCK, VIP and SP were applied locally by iontophoresis and/or pressure microinjection. An excitability testing procedure of single sural afferent C- and A-fibers was used to study possible presynaptic actions of NE, SS and L-glutamate applied locally (iontophoresis and/or pressure microinjection) at the intraspinal sites of minimal threshold for their antidromic activation. It was believed that this study will provide new information about the possible functional role of these agents in synaptic transmission processes in the mammalian spinal dorsal horn.

Literature Review

Dorsal horn - structural considerations

Until recently, there was conflicting information regarding the mode in which the primary afferent fibers of different size and functional properties terminate in the spinal cord. Although a number of morphological (Ranson, 1913; Earle, 1952; LaMotte, 1977) and physiological studies (Christensen and Perl, 1970; Kumazawa and Perl, 1978) suggested

that the small diameter afferent fibers terminate in the superficial dorsal horn, whereas large fibers are distributed to the nucleus proprius and deeper regions, there was a disagreement regarding their termination more superficially in the dorsal horn (Ramón y Cajal, 1909; Szentágothai, 1964; Sterling and Kuypers, 1967; Wall, 1967; Scheibel and Scheibel, 1968, 1969; Kerr, 1975). Recently, Light and Perl (1977a,b, 1979) utilized the technique of anterograde transport of horseradish peroxidase in dorsal rootlets to demonstrate details of the afferent fiber terminations in the dorsal horn of the monkey, cat and rat. They suggested that "the superficial dorsal horn can be divided into at least four regions: 1) The marginal zone (lamina I of cat) appears to receive terminations from intermediate (smaller myelinated) fibers; 2) the outer substantia gelatinosa (outer lamina II) receives many terminations from the very finest afferent fibers; 3) the inner substantia gelatinosa (inner lamina II) receives endings from some of the finest fibers and also from intermediate (smaller myelinated) fibers; and 4) the superficial part of the nucleus proprius (lamina III) receives endings from intermediate and large diameter dorsal root fibers." These conclusions are in a fairly good agreement with the results obtained by the Ralstons (Ralston, 1979; Ralston and Ralston, 1979) in their electron microscopic study of the distribution of dorsal root axons in laminae I-III of the macaque spinal cord using both degeneration and autoradiographic methods. LaMotte (1977) found a similar pattern of termination of primary afferents in the monkey's cervical spinal cord. However, Gobel and his colleagues (Gobel and Binck, 1977; Gobel and Hockfield, 1977; Gobel, 1978a,b) have reported that the smallest diameter afferents (C-fibers) in the trigeminal n. caudalis are distributed to the

marginal zone, whereas the larger diameter fibers (Aδ) projected to the gelatinosa region.

Purely on the basis of morphological data, it is difficult to speculate on the relative distribution of the smallest axons in the laminae I and II since Hamano et al. (1978) have observed fine arborizations of the collaterals of the larger myelinated fibers in this region. However, the data obtained with marking of the single functionally identified primary afferent fibers with horseradish peroxidase (Brown et al., 1976, 1977, 1978; Light and Perl, 1977a,b, 1979; Mense et al., 1981) have provided evidence for highly specific central projections of primary afferent fibers which appear to be related to sensory functions. Thus, Light and Perl (1979) found that the high-threshold mechanoreceptors (mechanical nociceptors) terminated in the marginal layer of the dorsal horn and in the ventral parts of the nucleus proprius (lamina V in the cat, see also Hamano et al., 1978). In addition, a smaller contralateral projection was found in the marginal zone and at the base of the nucleus proprius. In contrast low threshold mechanoreceptors (D-hair and field) had a different distributions of terminals; the D-hair fibers terminated in the dorsal part of the n. proprius including the inner part of the substantia gelatinosa whereas the field receptor fibers terminated principally in the middle part of the n. proprius.

Cytoarchitecture of the dorsal horn In his paper on cytoarchitectonic organization of the spinal cord in the cat, Rexed (1952) subdivided the dorsal horn into six laminae.

Lamina I (marginal zone) is represented by a narrow strip of neurons lying between the tract of Lissauer, also partly within the tract and the

substantia gelatinosa. Its most characteristic feature is the presence of moderate to large-sized marginal neurons (10-30 μm) horizontally oriented, with dendritic systems following the contours of the substantia gelatinosa (Waldeyer, 1888; Rexed, 1952; Scheibel and Scheibel, 1968; Light et al., 1979, 1981). The primary axonal afferents to lamina I are either direct collaterals of primary afferents or collaterals and/or terminals of axons running longitudinally in the dorsal and dorsolateral white matter. In addition, a second system of low density inputs may also arrive via the fine axonal outflow from the Lissauer's tract (Ramón y Cajal, 1909; Scheibel and Scheibel, 1968). There are three major projections from the marginal cells: 1) to the thalamus via the spinothalamic tract lying in the ventrolateral white matter on the contralateral side of the spinal cord (Kuru, 1949; Morin et al., 1951; Willis et al., 1974; Trevino and Carstens, 1975; Carstens and Trevino, 1978), 2) to the cerebellum via both the ipsilateral and contralateral lateral funiculi of the spinal cord (Snyder et al., 1978a), and 3) to other regions of the spinal cord via propriospinal pathways (Lenhossék, 1895; Ramón y Cajal, 1909; Szentágothai, 1964; Burton and Loewy, 1976). At least two other cell types are identified in lamina I whose functions are less well-understood. One of these is a very small cell which resembles gelatinosa neuron, while the other is a medium-sized neuron found at the junction of laminae I and II and named "limiting cell" (Ramón y Cajal, 1909).

Lamina II (substantia gelatinosa) is characterized by its pale appearance due to the paucity of myelinated axons and the abundance of small cells (6-15 μm in diameter). It is located ventrally to Lamina I. It contains medium-sized and small neurons (Earle, 1952; Pearson, 1952; Rexed,

1952, 1954; Réthelyi and Szentágothai, 1969, 1973; Sugiura, 1975; Mannen and Sugiura, 1976; Beal and Cooper, 1978; Gobel, 1978b). Ramón y Cajal (1909) described two major cell types in lamina II: 1) the central cells and 2) the border cells. Several investigators have recently recorded activity from the central cells, both extra- and intracellularly (Cervero et al., 1977; Hentall, 1977; Yaksh et al., 1977; Wall et al., 1979). On the basis of their axonal projections, the central cells can be subdivided into two groups: 1) funicular cells and 2) short-axoned cells. The funicular cells send their axons either into the Lissauer's tract or the dorsolateral fasciculus proprius. The axons of the short-axoned cells end within the substantia gelatinosa (Ramón y Cajal, 1909; Szentágothai, 1964; Scheibel and Scheibel, 1968; Matsushita, 1969; Sugiura, 1975; Czarkowska et al., 1976; Mannen and Sugiura, 1976; Snow et al., 1976). Substantia gelatinosa was thought for a long time to represent a closed system where the cells project only for a distance of a few spinal segments and terminate in the substantia gelatinosa of the same and contralateral side. However, recent studies demonstrated that at least some neurons of the substantia gelatinosa project to the lower brain stem (Giessler et al., 1978) and contralateral thalamus (Willis et al., 1978). The neurons belonging to long projection pathways include the central cells and the border cells.

Lamina III can be distinguished from L-II by somewhat larger neurons which are less closely packed than in L-II. Although axonal projections of L-III cells appear to resemble the axonal projections of L-II cells in ending in the substantia gelatinosa, some axons travel widely through the deeper laminae of the dorsal horn before re-entering the substantia gela-

tinosa (Matsushita, 1969, 1970; Mannen and Sugiura, 1976). Primary afferent input to this region comes principally from the coarse myelinated primary afferent fibers (Ralston, 1965, 1979; Sterling and Kuypers, 1967; Shriver et al., 1968; Réthelyi, 1977; Light and Perl, 1977a,b, 1979). Additional support for this statement comes from the studies of Brown and his colleagues who injected coarse primary afferents with horseradish peroxidase and demonstrated the presence of a marker in the flame-shaped arborizations of L-III (Brown, 1977; Brown et al., 1977).

Laminae IV-VI or nucleus proprius represent the bulk of the dorsal horn lying deep to the substantia gelatinosa. This nucleus proprius portion of the dorsal horn is characterized by having much larger neurons than the substantia gelatinosa and a large number of myelinated fibers. The neurons appear to increase in size from lamina IV to VI. The major dendritic arbor of lamina IV neurons is directed dorsally, often but not always reaching lamina II (Szentágothai, 1964; Brown et al., 1976; Proshansky and Egger, 1977). Szentágothai (1964) gave special emphasis to the cells with dendrites predominantly dorsally oriented and called these the "antenna-type neurons." The dendritic organization of lamina V and VI cells is not markedly different from the dendritic organization of the cells in lamina IV. The cells from the nucleus proprius are thought to project to the lateral cervical nucleus, the cerebellum, the thalamus and other areas of the spinal cord (Ramón y Cajal, 1909; Brown and Franz, 1969, 1970; Matsushita, 1969, 1970; Willis et al., 1974, 1979; Mannen, 1975; Snyder et al., 1978a). There is a major shift in orientation of the primary afferents at the junction of lamina IV and V, the orientation changing from longitudinal to dorsoventral (Sprague and Ha, 1964; Sterling

and Kuypers, 1967; Scheibel and Scheibel, 1968; Szentágothai and Réthelyi, 1973). The primary afferent input to lamina VI is complex. Many collaterals from primary afferent axons destined to reach ventral horn cells end in this area (Ramón y Cajal, 1909; Scheibel and Scheibel, 1969).

Dorsal horn - functional considerations

Interest in the functional role of the marginal zone and substantia gelatinosa increased markedly after Perl and his collaborators showed that neurons in these two layers are excited in a specific way by noxious mechanical and thermal stimuli carried by fine myelinated and unmyelinated fibers (Christensen and Perl, 1970; Kumazawa and Perl, 1976, 1978). In extracellular recordings from lamina I neurons of the cat (Christensen and Perl, 1970) and the monkey (Kumazawa and Perl, 1976) three different kinds of neurons, as determined on the basis of their responses to natural stimuli, were described: (1) neurons responding only to intense mechanical stimulation of the skin, (2) neurons responding to intense mechanical and thermal stimulation of the skin and (3) neurons responding to intense mechanical and thermal stimuli and to innocuous temperature changes of the skin. These results are largely confirmed but wide-dynamic-range (WDR) neurons, activated by both low and high threshold inputs, were also described (Cervero et al., 1976; Price et al., 1979). In addition, some neurons in the marginal layer were excited by electrical stimulation of group III and IV muscle afferent fibers (Cervero et al., 1976), and some were excited polysynaptically from Aβ-mechanoreceptive afferent fibers (Price et al., 1979).

The functional properties of most neurons in substantia gelatinosa

are similar to those in the marginal layer. In addition to nociceptive and thermoreceptive cells, substantia gelatinosa neurons were found to be activated by slowly conducting myelinated and/or unmyelinated afferent fibers that were maximally excited by innocuous mechanical stimuli (Kumazawa and Perl, 1976, 1978; Yaksh et al., 1977; Wall et al., 1979). Price et al. (1979) described three classes of neurons in this layer: (1) low threshold mechanoreceptive (LTM) neurons, responded maximally to gentle mechanical stimulation (light brushing, hair movement), (2) wide-dynamic-range neurons responded to nociceptive and to innocuous low-threshold input and (3) nociceptive specific (NS) neurons responded to noxious mechanical and thermal stimulation only. In the study of substantia gelatinosa neurons, Cervero et al. (1977, 1979) established a set of criteria to separate activity generated by substantia gelatinosa neurons from that produced by other dorsal horn cells. The majority (86%) of their cells had spontaneous activity and an inhibitory cutaneous receptive field and were placed in a class called "inverse;" remainder of the cells were placed in a class called "heterogeneous." According to these studies, "inverse" neurons are the mirror image of the larger neurons from deeper laminae of the dorsal horn and could be classified into three sub-groups: (1) "inverse" 1 ($\bar{1}$) inhibited only by innocuous stimulation of the skin, (2) "inverse" 2 ($\bar{2}$) inhibited by both nocuous and innocuous stimulation and (3) "inverse" 3 ($\bar{3}$) inhibited only by nocuous stimulation. The "heterogeneous" group represents neurons with unusual physiological properties such as rapid habituation, activity long outlasting sensory input or very small receptive field. The substantia gelatinosa neurons with unusual physiological properties are described by other investigators (Hentall,

1977; Yaksh et al., 1977; Price et al., 1979; Wall et al., 1979).

Wall (1960) demonstrated that cells in the nucleus proprius typically responded to activation of low and high intensity mechanical stimuli. Wagman and Price (1969) often demonstrated a convergence of Aα, Aβ, Aδ and C fibers upon the same neuron. In addition, it has been shown that neurons in the deeper laminae (VI and VII) receive an input from high threshold muscle afferents (Eccles et al., 1956, 1960). Wall (1967) found that interneurons in the nucleus proprius have different response properties in different laminae. Cells in lamina IV were excited by low threshold stimuli (hair movement, touch and stimulation of tactile dome) and some were excited additionally by pressure and pinch. Cells in lamina V and VI were mostly of the WDR type. However, it has been shown that there are neurons in the nucleus proprius (mostly lamina V) which respond only to high intensity painful stimuli (Kolmodin and Skoglund, 1960; Gregor and Zimmermann, 1972; Price and Browe, 1973; Menétrey et al., 1977).

Dorsal horn - pharmacological considerations

Cholecystokinin Cholecystokinin (CCK), first purified from gut extracts as a peptide composed of 33 amino acids (Mutt and Jorpes, 1971) was later shown to be present in the central nervous system (CNS) and the peripheral nervous system (PNS) (Dockray et al., 1978; Larsson and Rehfeld, 1979; Rehfeld et al., 1979). Both in the gut and in the brain, CCK was found to consist of five main components of which the C-terminal octa- (CCK-8) and tetrapeptides (CCK-4) were the most abundant (Dockray et al., 1978; Rehfeld, 1978).

Within the mammalian CNS and PNS, CCK is unevenly distributed (Rehfeld, 1978; Larsson and Rehfeld, 1979). Highest CCK-like immunoreactivity is found in the neocortex, limbic system and dorsal horn of the spinal cord (Larsson and Rehfeld, 1979; Rehfeld et al., 1979; Yaksh et al., 1982). Immunohistochemical studies have localized CCK-8-like immunoreactivity to nerve cell bodies, fibers and nerve endings where it occurs in the synaptic vesicles (Pinget et al., 1978; Larsson and Rehfeld, 1979; Emson et al., 1980a). In the spinal cord of cat, rat, guinea-pig, hog and man the CCK-immunoreactivity was detected in the superficial laminae of the dorsal gray matter and around the central canal (lamina X) at all levels of the spinal cord, as well as in the spinal ganglia (Larsson and Rehfeld, 1979; Rehfeld et al., 1979; Gibson et al., 1981; Yaksh et al., 1982). In addition to small CCK-containing fibers, CCK-containing cell bodies from lamina II to upper lamina V in the rat spinal dorsal horn were observed (Gibson et al., 1981). Hökfelt (1979) estimated that about 10-20% of the total population of dorsal root ganglia neurons contain CCK-like peptide. Unilateral rhizotomies of dorsal roots L4 to S1 produced a severe depletion of CCK-8 in the dorsal horn of the lesioned as compared to the non-lesioned side, while cervical hemisection had no effect (Yaksh et al., 1982). These findings suggest axoplasmic transport of the peptide from the spinal ganglia to the axon terminals in the spinal cord.

Rehfeld et al. (1979) have been able to show an extensive and rapid in vivo biosynthesis of CCK in the neurons of the rat cerebral cortex. Furthermore, they found that CCK synthesis involves precursor forms which are processed by further cleavage to the putative transmitter forms - the octapeptide and/or the tetrapeptides. Malesci et al. (1980) found that

there are at least two enzymes in porcine cerebral cortical tissue involved in the conversion of CCK to CCK-12 and CCK-8.

The Ca^{2+}-dependent mechanism of CCK-8 release by depolarizing stimuli from the brain slice preparations and synaptosomes has been shown (Pinget et al., 1979; Rehfeld et al., 1979; Dodd et al., 1980; Emson et al., 1980b). In addition, a significant increase in the level of CCK-8 in the spinal perfusate as a result of electrical stimulation of the sciatic nerve was observed by Yaksh et al. (1982). In the brain regions high in CCK content, the peptide binds specifically and reversibly to the high affinity, saturable, receptor sites (Saito et al., 1980; Innis and Snyder, 1980). Furthermore, Innis and Snyder (1980) demonstrated that CCK receptors in the brain differ markedly from CCK receptors in the pancreas.

Effects of CCK on central neurons have been studied both in vivo and in vitro. After intracerebroventricular injection in sheep CCK-8 produced satiety (Della-Fera and Baile, 1979). When CCK-8 was administered subcutaneously in mice (Zetler, 1980) as well as into the brain and the lumbar subarachnoidal space of rats (Jurna and Zetler, 1981), it produced naloxone-sensitive antinociceptive effects. Iontophoretic application of CCK-7 onto single neurons in the areas A9 and A10 of the rat brain produced an increase in their firing rate and induced bursting activity (Bunney et al., 1980). Similar excitatory action of the CCK-8 has been observed in the spinal dorsal horn neurons in vivo and in vitro (Jeftinija et al., 1980, 1981a). In addition, Phillis and Kirkpatrick (1979b) have shown that the application of CCK to the isolated hemisected toad spinal cord causes a tetrodotoxin-(TTX)-sensitive depolarization of motoneurons and dorsal root terminals. The same authors (1980) showed that CCK-8 excited

28% of the unidentified neurons and 22% of the corticospinal neurons in the rat sensory-motor cerebral cortex. The excitatory, depolarizing action of CCK was observed by Dodd and Kelly (1979, 1981) and Kelly and Dodd (1981) in pyramidal cells in the rat hippocampal slice preparation *in vitro*.

Until now, no specific antagonist for CCK is known. However, Zetler (1979) has shown antagonism of CCK by opioid peptides in the isolated guinea-pig ileum.

Somatostatin Somatostatin (SS-14) is a cyclized tetradecapeptide (H_2N-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) originally isolated from ovine hypothalami as the growth hormone release inhibiting factor (GHRIF; Brazeau et al., 1973; Burgus et al., 1973). However, recently Brazeau et al. (1981) have isolated, from the extract of ovine hypothalami, two larger molecular weight somatostatins, SS-28 and SS-25, which are more potent than SS-14 in inhibiting the secretion of the growth hormone both *in vitro* and *in vivo*. In addition, they showed that synthetic forms of SS-28 and SS-25 possess biological activity.

Using radioimmunoassay (Brownstein et al., 1975), bioassay and immunohistochemical procedures (Dubois et al., 1974; Pelletier et al., 1974; Hökfelt et al., 1975a, 1976; King et al., 1975; Sétáló et al., 1975; Alpert et al., 1976) it has been shown that SS is widely distributed throughout the mammalian CNS. In the rat, Hökfelt et al. (1975a, 1976) demonstrated the presence of SS in small neuronal cell bodies of the spinal ganglia (about 10% of the total population). In addition, SS positive fibers were observed in the dorsal horn (the highest concentration being in lamina II), Lissauer's tract, and adjacent areas of the lateral funiculus.

Similarly to CCK not much is known about SS biosynthesis. Several investigators observed the existence of peptides of larger molecular weight having SS immunoreactivity and concluded that these polypeptides might be SS precursors (Zingg and Patel, 1979; Zyznar et al., 1979). Recently, Pradayrol et al. (1980) isolated and analyzed the sequence of a 28 amino acid polypeptide with a COOH-terminal identical to SS-14. However, Brazeau et al. (1981) demonstrated that SS-28 is biologically more active than SS-14 and thus challenged the possibility that SS-28 is a precursor form.

The voltage- and calcium-dependent release of SS, from primary sensory neurons in the dissociated cell cultures (Mudge et al., 1977) and from primary afferent fibers of the intact rat and cat spinal cord has been demonstrated (Jessell et al., 1979a).

Action of SS has been studied both in vivo and in vitro. Applied by iontophoresis, SS depressed spontaneous and/or evoked activity in cerebral cortical and hypothalamic neurons (Renaud et al., 1975). Similar depressant action of SS on the excitability of nociceptive dorsal horn neurons located in laminae I, II and V of the intact cat spinal cord (Randić and Miletić, 1978) and on dorsal horn neurons in the rat spinal cord slice preparation (Miletić and Randić, 1981) has been shown, as well as in motoneurons and primary afferent fibers of the frog spinal cord (Padjen, 1977). However, in the neurons of frontal and parietal cortex (Ioffe et al., 1978) hippocampus and striatum (Olpe et al., 1980), as well as corticospinal tract (Phillis and Kirkpatrick, 1980) SS was shown to be mostly excitatory. In agreement with the latter findings, Dodd and Kelly (1978) observed that iontophoretically applied SS strongly excited some pyramidal neurons in the rat hippocampal slice. This excitation was accompanied by a depolarization

of the neuronal membrane without any change in the membrane input resistance. However, in mouse spinal cord neurons grown in culture, Macdonald and Nowak (1981b) found that SS-produced depolarization was associated with a decrease in membrane conductance. In contrast, a reversible, dose-dependent hyperpolarization of hippocampal (Pittman and Siggins, 1981) and dorsal horn neurons (Murase and Randić, 1981; Murase et al., 1982) was a predominant finding when SS was applied into the bathing medium. The hyperpolarization was usually accompanied by a decrease in membrane input resistance. Besides acting at postsynaptic sites, a presynaptic site of action of SS was observed on cultured spinal cord neurons (Macdonald and Nowak, 1981a) and dorsal root ganglion cells (Dunlap and Fischbach, 1978). In the latter case, SS was found to decrease the calcium component of neuronal action potential. A presynaptic site of action of SS was also suggested by our experiments, showing that iontophoretically applied SS increases excitability of some cutaneous primary afferent C- and Aδ-fibers (Jeftinija and Randić, 1981).

In addition to the above effects, SS has been shown to specifically depress responses of glutamate, but not GABA, in both dorsal and ventral roots of the frog spinal cord (Padjen, 1977), and to augment glutamate responses in the sensorimotor cortex of awake rabbits (Ioffe et al., 1978). When SS was applied together with glutamate, the responses of rat cortical neurons in culture to glutamate were specifically enhanced (Dichter and Delfs, 1981). In the latter case, SS enhanced the presynaptic effect of glutamate and potentiated its postsynaptic action. Thus, it appears that SS may play a role of a neuromodulator in mammalian cerebral cortex by potentiating the action of glutamate.

Göthert (1980) recently reported that SS selectively inhibits norepinephrine release from the rat hypothalamus leaving the release of dopamine and serotonin from this tissue unaffected. The presynaptic site of action was suggested because the effect was also observed in the presence of TTX. However, Tanaka and Tsujimoto (1981) reported that SS facilitates voltage- and K^+-evoked release of serotonin from the cerebral cortex, hippocampus and hypothalamus. These results suggest a functional connection between the monoaminergic pathways and the SS-containing neurons in the CNS.

The physiological role of SS-containing primary afferent neurons is at present unknown. One of the possibilities is that SS may have the role of an inhibitory transmitter at this site. Although inhibitory primary sensory neurons have so far not been reported, inhibitory mechanisms involved in modulation of the transmission of nociceptive information were postulated (Melzack and Wall, 1965).

No known specific antagonist of SS actions has been found to date.

Substance P Substance P (SP) is an undecapeptide (H_2N-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-OH) discovered in extracts of horse brain and intestine by von Euler and Gaddum (1931), purified by Chang and Leeman (1970) and synthesized by Tregear et al. (1971). However, neither biosynthesis of SP in the mammalian brain nor its mode of degradation have yet been fully characterized.

In the central nervous system, SP distribution is uneven. The highest levels are found in dorsal roots and superficial parts of the spinal dorsal horn (Lembeck, 1953; Pernow, 1953; Hökfelt et al., 1975b, 1976; Chan-Palay and Palay, 1977; Pickel et al., 1977; Cuello and Kanazawa, 1978; Barber et

al., 1979), trigeminal nucleus, pars reticularis of the substantia nigra, hypothalamus and interpeduncular nucleus (Ljungdahl et al., 1978). SP-positive fluorescence was found in about 20% of the small dorsal root ganglion cells, and probably unmyelinated or thinly myelinated axons in the superficial parts of the dorsal horn (Hökfelt et al., 1975b, 1977; Chan-Palay and Palay, 1977). Transection of the dorsal roots results in a marked decrease in the number of SP-positive fibers in the SG but not in the ventral horn (Jessell et al., 1979b). The latter result supports the view that SP-positive fibers in laminae I-III originate in the spinal ganglia and that there is a transport of SP within the central branch of the spinal ganglion cells. Using the peroxidase-antiperoxidase method, SP was found to be localized in the synaptic terminals (Chan-Palay and Palay, 1977; Hökfelt et al., 1977; Pelletier et al., 1977; Pickel et al., 1977; Barber et al., 1979). Within the SP-containing terminals, SP was found to be associated both with the large granular and small agranular vesicles (Chan-Palay and Palay, 1977; Barber et al., 1979). This finding is of interest since it indicates that these terminals may contain more than one transmitter.

The early studies of Jancsó and his collaborators on capsaicin, a homovanillic acid derivative (8-methyl-N-vanillyl-6-noneamide), have shown that it stimulates chemosensitive pain receptors in the periphery and produces complete insensitivity to all kinds of painful stimuli after repeated administration (Pórszász and Jancsó, 1959; Jancsó et al., 1968). It has also been shown that systemic treatment of adult animals with capsaicin induces the depletion of SP from primary afferent fibers in the dorsal horn of the spinal cord (Gašparović et al., 1964; Jessell et al.,

1978). Ca^{2+}-dependent capsaicin-induced release of SP from spinal cord in vivo (Yaksh et al., 1979) and in vitro (Gamse et al., 1979; Theriault et al., 1979) has been shown. Capsaicin given to the newborn rats causes both nerve terminal degeneration (Jancsó et al., 1977) and a permanent reduction of SP in the spinal dorsal horn (Gamse et al., 1980; Nagy et al., 1980). The degeneration of afferent fibers and SP depletion are associated by a prolonged insensitivity of the treated animals to nociceptive stimuli (Jancsó et al., 1977; Holzer et al., 1979). These results suggest that SP may be a transmitter of the pain fibers. Recently, however, Gamse et al. (1981a,b) demonstrated that capsaicin can release and deplete SP as well as SS from primary sensory neurons. These findings by Gamse et al. (1981a,b) suggest that capsaicin has multiple actions on primary sensory neurons and that the action of capsaicin is not specific for SP-containing fibers.

The Ca^{2+}-dependent release of SP-like immunoreactivity from the isolated rat spinal cord in response to the stimulation of the dorsal roots and high K^+ (55 mM) has been demonstrated (Otsuka and Konishi, 1976), as well as in the cultures of dissociated dorsal root ganglia (Mudge et al., 1977). Release of SP (K^+ or capsaicin-evoked) has also been reported from the mammalian spinal cord in vivo (Jessell et al., 1979a). In addition, the electrical stimulation of the sciatic nerve evoked the release of SP only, when stimulus parameters were of such intensities as to activate small myelinated (Aδ) and unmyelinated (C) fibers (Jessell et al., 1979a). The latter finding provides further support for the presence and release of SP from the small diameter afferent fibers.

Actions of SP on the spinal neurons have been studied both in vitro

and *in vivo*. In the *in vitro* experiments, Otsuka and his collaborators demonstrated that exogenously applied SP has a direct depolarizing effect on both frog motoneurons (Otsuka et al., 1972) and motoneurons of the newborn rat (Konishi and Otsuka, 1974). In the neonatal rat spinal cord slice preparation, SP depolarized dorsal horn neurons and increased their excitability (Miletić and Randić, 1980b, 1981; Murase and Randić, 1981; Murase et al., 1982). Similar depolarization produced by SP has been observed in dissociated mammalian spinal neurons grown in tissue culture Barker et al., 1980; Hösli et al., 1981; Nowak and Macdonald, 1981).

Iontophoretic application of synthetic SP has a strong, although slow excitatory action on dorsal horn interneurons in the intact cat spinal cord and cuneate nucleus (Krnjević and Morris, 1974; Henry et al., 1975; Henry, 1976; Randić and Miletić, 1976, 1977). Subsequent intracellular recordings from dorsal horn units and motoneurons *in vivo* confirmed a potent and slow depolarization (Krnjević, 1977; Sastry, 1979b; Zieglgänsberger and Tulloch, 1979).

The mechanism of action of SP, however, remains uncertain. Depolarization evoked by SP has been associated with an increase (Nicoll, 1978; Otsuka et al., 1978), a decrease (Krnjević, 1977; Katayama and North, 1978; Grafe et al., 1979; Katayama et al., 1979; Murase et al., 1982) or no change (Sastry, 1979b; Zieglgänsberger and Tulloch, 1979) in membrane conductance. The membrane conductance increase was suggested to be due to an increase in sodium conductance (Nicoll, 1978; Otsuka et al., 1978) while decreased potassium conductance (Krnjević, 1977; Katayama and North, 1978; Katayama et al., 1979; Hösli et al., 1981; Nowak and Macdonald, 1981) or chloride conductance (Krnjević, 1977) was thought to be responsible for

an increase in neuronal membrane input resistance.

The slow and long-lasting nature of the SP response, as well as its modulation of glutamate effects, have led Krnjević to suggest that SP is more likely to be a regulator of neuronal excitability than an actual transmitter involved in modulating sensory inputs (Krnjević and Morris, 1974). Secretion of SP thus, may be involved in a long term regulation of synaptic efficacy. In agreement with this hypothesis is the finding that SP terminals in the dorsal horn of the spinal cord contain a large number of small round vesicles with SP-positive material on the outside of these vesicles and unknown material inside (Barber et al., 1979).

Although intracellular recordings revealed that SP depolarizes dorsal horn neurons, the result indicative of its postsynaptic action (Sastry, 1979b; Zieglgänsberger and Tulloch, 1979; Murase et al., 1982), an additional presynaptic site of action has been suggested by the following data. SP has been reported to depolarize amphibian primary afferent fibers (Nicoll, 1976), to reduce synaptic efficacy at the Mauthner fiber-giant fiber synapse of the hatchetfish (Steinacker and Highstein, 1976), to have a strong presynaptic effect on transmission at the frog neuromuscular junction (Steinacker, 1977), to modify neurotransmitter release in spinal cord neurons grown in culture (Nowak and Macdonald, 1981), and to produce dual effects on the excitability of single cutaneous primary afferent C- and A-fibers in the cat spinal cord (Randić, 1981).

The lack of a selective SP antagonist was a serious handicap in evaluating the neurotransmitter role of SP. Several substances, some of them SP analogs, have been employed in attempts to specifically antagonize the peptide's actions. The claim by Saito et al. (1975) that Lioresal

[β-(4-chlorophenyl)-γ-aminobutyric acid], an analogue of GABA which has been used in treatment of spasticity, abolishes both the action of SP, and the spinal monosynaptic reflex in the newborn rat, has been challenged (Henry and Ben-Ari, 1976). However, it has been recently reported that the SP-analog, (D-Pro^2, D-Phe^7, D-Trp^9)-SP, specifically antagonizes the smooth muscle stimulating effect of SP, inhibites the SP-induced salivary secretion, and abolishes the vasodilatory effect of SP (Folkers et al., 1981; Rosell et al., 1981a). Furthermore, Rosell et al. (1981b) have found that three specific SP-analogs [(D-Pro^2, D-Phe^7, D-Trp^9)-SP, (D-Pro^2, D-Trp^{7,9})-SP and (D-Arg^1, D-Pro^2, D-Phe^7, D-Trp^9)-SP] blocked SP-induced contraction of the isolated guinea-pig ileum and some also inhibited SP-evoked salivary secretion. Of a particular interest was the finding that (D-Pro^2, D-Trp^{7,9})-SP antagonizes SP-induced excitation of the locus coeruleus neurons in a specific manner (Engberg et al., 1981a,b).

Vasoactive intestinal polypeptide Vasoactive intestinal polypeptide (VIP) is a 28 amino acid residue peptide originally isolated from porcine duodenum by Said and Mutt (1970a,b). Its amino acid sequence was subsequently established (Mutt and Said, 1974). Cytochemical and radioimmunochemical studies have revealed the presence of VIP in neuronal cell bodies and terminals of the mammalian CNS and PNS (Bryant et al., 1976; Larsson et al., 1976b; Fuxe et al., 1977; Besson et al., 1978; Emson et al., 1978; Fahrenkrug and Schaffalitzky de Muckadell, 1978; Lundberg et al., 1978, 1979; Schultzberg et al., 1978; Palkovits et al., 1981; Yaksh et al., 1982). Immunohistochemical studies of the distribution of VIP-containing neurons in the brain have shown that high levels of this peptide are present in the cerebral cortex, amygdala, hypothalamus

and cerebrovascular nerves (Larsson et al., 1976a; Fuxe et al., 1977; Emson and Lindvall, 1979; Lorén et al., 1979; Emson et al., 1980a; Sims et al., 1980). VIP-positive neurons are also found in the spinal dorsal root ganglia (Lundberg et al., 1978), where approximately 5-10% of all ganglion cells contain VIP-like immunoreactivity (Hökfelt, 1979). In the rat spinal dorsal horn, VIP-positive fibers are concentrated in laminae I and II, some are also found around the central canal (lamina X) and a few in lamina VII (Lundberg et al., 1979; Gibson et al., 1981). Unilateral rhizotomies of the lumbosacral dorsal roots but not the cervical hemisection reduced the levels of VIP in the dorsal horn significantly (Yaksh et al., 1982). The latter finding is consistent with the view that VIP-positive fibers in the superficial dorsal horn originate in the spinal ganglia and that there is a transport of VIP within the central branch of the spinal ganglion cells. The peripheral transport of VIP from spinal dorsal root ganglia has also been reported (Lundberg et al., 1978). It appears that the spinal cord does not contain any intrinsic VIP-positive neurons (Emson et al., 1979). Within the VIP-containing terminals the peptide was found to be associated with vesicles (Giachetti et al., 1977; Emson et al., 1978). The voltage and Ca^{2+}-dependent release of VIP from the cortical synaptosomes (Giachetti et al., 1977), hypothalamic slices (Emson et al., 1978) and into the spinal perfusate of intact cats (Yaksh et al., 1982) has been demonstrated. The experiments in cats showed that electrical stimulation of the sciatic nerve evoked release of VIP, only when stimulus parameters were of such intensities to activate small myelinated (Aδ) and unmyelinated (C) fibers (Yaksh et al., 1982).

Action of VIP on central neurons has been studied both in vivo and

in vitro. Phillis and his collaborators have shown that iontophoretically applied VIP excited spontaneously active rat cerebral cortical neurons (Phillis et al., 1978; Phillis and Kirkpatrick, 1980), and depolarized motoneurons and dorsal root terminals when applied topically to the toad spinal cord (Phillis et al., 1978; Phillis and Kirkpatrick, 1979b). VIP was found to be very potent excitant of neurons throughout rat trigeminal nucleus caudalis when applied iontophoretically (Salt and Hill, 1981). VIP also excited CA1 neurons in the rat hippocampus which effect was accompanied by a depolarization and a large increase in membrane conductance (Dodd et al., 1979). Applied iontophoretically and/or by pressure microinjection, VIP caused a strong excitation of a majority of the tested dorsal horn interneurons in the cat spinal cord; while in the spinal cord slice preparation, VIP applied in the bath depolarized dorsal horn neurons and increased their excitability (Randić and Jeftinija, 1981; Jeftinija et al., 1982a).

No detailed pharmacology of the interaction of VIP with its receptor has been carried out. A VIP-sensitive adenylate cyclase has been demonstrated in the brain (Deschodt-Lanckman et al., 1977; Quik et al., 1979) and in the retina (Schorderet et al., 1981).

No known specific antagonist of VIP has been found to date, although Lundberg et al. (1980) reported that avian pancreatic polypeptide markedly reduced VIP-induced vasodilatation in the cat submandibular salivary gland. The exact mechanism for this inhibition has yet to be established.

L-glutamate L-glutamate, discovered by Ritthausen in 1866, has, since the beginning of the century, been known as an important constituent of the nervous tissue (Abderhalden and Weil, 1913). It is present in the

brain in higher concentration (10^{-2} M) than in most other organs. The possibility that glutamate might be concerned with the control of neuronal excitability was first proposed by Hayashi (1954) who demonstrated the excitatory effect of glutamate on the cerebral cortical neurons.

Variations in distribution of L-glutamate in the mammalian central nervous system have been observed with a stepwise increase in its content in the gray matter when progressing from lower to higher areas. This distribution pattern may reflect different functional roles of this amino acid in metabolic or synaptic processes (Johnson and Aprison, 1971). In many brain areas it has proven difficult to correlate the levels of L-glutamate with its putative neurotransmitter role. An exception is, however, the spinal cord, where L-glutamate is more concentrated in the dorsal than in the ventral roots, and the highest level was found to be in the dorsal gray area where primary afferent fibers terminate (Graham et al., 1967). Johnson and Aprison (1970) confirmed the latter finding and in addition demonstrated a lower L-glutamate level in the distal sensory root compared to the proximal dorsal root or the ganglion, the fact suggesting a specific transport mechanism for L-glutamate towards the spinal cord where synaptic contacts are made.

Evidence for vesicular storage of putative transmitter amino acids had long been sought without success. An enrichment of glutamate and aspartate, along with acetylcholine and GABA, was recently reported in the vesicular fraction from the bovine cerebral cortex (Zisapel and Zurgil, 1979). However, in another study, no such enrichment was found in vesicles from several different brain regions (Kontro et al., 1980).

At least two kinetically distinct transport systems have been observed for L-glutamate in the CNS (Logan and Snyder, 1972). The "high affinity" system seems to be associated with distinct population of nerve terminals (Wofsey et al., 1971; Vincent and McGeer, 1980). Neither system is specific for L-glutamate, it may transport a number of acidic amino acids including L-aspartate (Balcar and Johnston, 1972).

The release of L-glutamate in response to depolarizing stimuli has been studied both *in vivo* and *in vitro*. Increased release of endogenous L-glutamate *in vivo* from the surface of the cat cerebral cortex has been observed during brain stem stimulation (Jasper and Koyama, 1969). Voltage and K^+-induced release of exogenous L-glutamate *in vitro* from slices of rat brain and spinal cord has also been observed (Wheeler et al., 1966; DeFeudis, 1971; Hammerstad and Cutler, 1972; Roberts and Mitchell, 1972; Roberts, 1974a).

L-glutamate was found to excite cat central neurons possibly by a reversible depolarization accompanied by an increase in membrane conductance (Curtis et al., 1960; Krnjević and Schwartz, 1967; Zieglgänsberger and Puil, 1973a). It appears, however, from the most recent work that the mechanism of excitation of central neurons by glutamate remains unclear. Thus, in an extensive study of L-glutamate action in cat spinal motoneurons, Engberg et al. (1979) found that conductance changes during amino acid induced excitation are dose-dependent. Thus, small glutamate responses were associated with either a small increase, or decrease or no change in neuronal input resistance. During large responses biphasic changes in membrane conductance were noted, i.e. the membrane conductance decreased during the early part of the depolarizing response and thereafter

increased. They suggested that the high conductance state is due to an increase in the glutamate uptake mechanism.

Since the initial studies in which amino acids were applied topically to the feline (Eccles et al., 1963b) and toad (Schmidt, 1963) spinal cord, it becomes clear that L-glutamate has a depolarizing effect on primary afferent terminals in addition to an already proven effect on post-synaptic neuronal elements. Curtis and Ryall (1966) tested the effect of iontophoretically applied L-glutamate on the electrical excitability of large primary afferent fibers, and found that L-glutamate reversibly increased terminal excitability of cutaneous and muscle afferents. The finding of De Groat et al. (1972) that L-glutamate does not modify excitability of dorsal root ganglia, as well as the reported observations of the depolarizing action of L-glutamate on the primary afferent neurons, in both mammalian and amphibian species (Barker et al., 1975; Curtis et al., 1977; Evans, 1978), lead to the conclusion that receptors for L-glutamate are confined exclusively to the central terminals of primary afferent neurons. However, on the basis of his data in the frog Evans (1980) proposed an alternative explanation of the depolarizing effect of L-glutamate on primary afferent fibers, namely that they do not possess excitatory amino acid receptors but are depolarized indirectly through the release of K ions from excited dorsal horn neurons. Similarly, Jahr and Nicoll (1981) gave serious consideration to an increase in extracellular potassium as being the causative factor in the significant depolarization produced by L-glutamate in the frog olfactory nerve in vitro.

Investigations into the possibility that L-glutamate is neuro-transmitter are in a large degree complicated by the absence of a specific

antagonist. Among the various potential L-glutamate antagonists are L-glutamic acid diethylester (GDEE) (Haldeman et al., 1972) and 1-hydroxy-3-aminopyrolidone-2 (HA-966) (Davies and Watkins, 1973), however specificity of these compounds was challenged (Curtis et al., 1972, 1973; Zieglgänsberger and Puil, 1973b). More recently, long chain mono- and diamino dicarboxylic acids were recommended as more specific antagonists of L-glutamate (Biscoe et al., 1977; Hall et al., 1977). But again, none of these compounds has specific antagonistic effect for L-glutamate, all of them antagonize effects of other excitatory amino acids to some extent (Biscoe et al., 1977; Evans et al., 1978). For more details on L-glutamate receptors classification see review by Watkins and Evans (1981). It is of interest that, the results obtained in these pharmacological studies, and also the results from binding studies suggest the existence of multiple receptive sites for L-glutamate (Michaelis et al., 1974; Roberts, 1974b) in the CNS.

5-Hydroxytryptamine (serotonin) The presence of serotonergic axons in the dorsal horn was first demonstrated by Dahlström and Fuxe (1965). Their origin is most probably in the cell groups of the midbrain raphe nuclei which have been demonstrated to contain 5-hydroxytryptamine (5-HT) (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Oliveras et al., 1977; Jacobowitz and MacLean, 1978; Pointras and Parent, 1978). Many neurons of the midbrain raphe nuclei project to spinal levels and terminate in the dorsal horn (Brodal et al., 1960; Kuypers and Maisky, 1975; Basbaum et al., 1978; Martin et al., 1978). Electrical stimulation near the 5-HT-containing neurons of the brain stem raphe region produces an analgesia in animals (Akil and Mayer, 1972; Oliveras et al., 1974, 1975;

Proudfit and Anderson, 1974) and inhibits spinal dorsal horn neurons that receive synaptic inputs from high threshold cutaneous afferents (Oliveras et al., 1974; Fields et al., 1977; Guilbaud et al., 1977; Willis et al., 1977; Duggan and Griersmith, 1979; McCreery et al., 1979). The mechanisms by which descending pathways activated by stimulation in the raphe nuclei inhibit spinothalamic tract cells and dorsal horn interneurons are uncertain. A prominent effect of iontophoretically applied 5-HT in the spinal cord is to depress the activity of the dorsal horn nociceptive neurons (Engberg and Ryall, 1966; Randić and Yu, 1976; Belcher et al., 1978; Griersmith and Duggan, 1980). In addition, the responses of the spinothalamic tract cells to pulses of glutamate are depressed by 5-HT, suggesting that 5-HT may have a postsynaptic action (Jordan et al., 1978). However, presynaptic mechanisms may also be involved (Carpenter et al., 1966; Proudfit and Anderson, 1974; Hentall and Fields, 1979; Carstens et al., 1981).

The first demonstration that a pharmacologically induced reduction in the concentration of brain 5-HT was associated with a change in the behavioral response to noxious stimuli was made by Tenen (1967). He found that animals pretreated with para-chlorophenylalanine (p-CPA), the drug that reduces the brain 5-HT content by inhibiting the enzyme tryptophan hydroxylase, had a decreased threshold for jump response when presented with an electroshock. The content of 5-HT is lowered to about 8% of the control level in the rat lumbar spinal cord two days after an i.p. injection of p-CPA (320 mg/kg) (Vogt, 1974).

Substance P and 5-hydroxytryptamine The coexistence of several putative neurotransmitters in a single neuron has been demonstrated in

invertebrates (Kerkut et al., 1967; Brownstein et al., 1974) and in the mammalian peripheral and central nervous systems (see Hökfelt et al., 1980). Recent reports suggest that 5-HT and SP are present in the same neurons in several parts of the CNS, especially in the region of the raphe nuclei and the dorsal raphe-spinal pathways (Chan-Palay et al., 1978; Hökfelt et al., 1978; Björklund et al., 1979; Chan-Palay, 1979; Singer et al., 1979). In both raphe nuclei and dorsal horn of the spinal cord, these two substances were found in the same large dense-core vesicles (Pelletier et al., 1981). Furthermore, it was found that intracysternal or intraventricular administration of chemical neurotoxins such as 5,6- or 5,7-dihydroxytryptamine into rats resulted in an almost complete disappearance of 5-HT immunofluorescence in both the dorsal and ventral horns. In addition, there was a marked depletion of SP-like immunoreactivity (SPLI) in the lumbar spinal ventral horn (Björklund et al., 1979) and only a slight decrease in SPLI in the dorsal horn (Hökfelt et al., 1978; Björklund et al., 1979).

In view of the data suggesting that SP may be the transmitter of nociceptive primary afferents, and that 5-HT in part mediates the descending inhibition of nociceptive transmission in the dorsal horn, it became of a particular interest recently to investigate functional significance of the "5-HT + SP" projection to the dorsal horn. Rivot et al. (1980) have found that descending inhibitory influences from nucleus raphe magnus (NRM) on responses of rat dorsal horn neurons to unmyelinated fiber inputs are reduced after 5-HT depletion by p-CPA and in addition long-lasting and sustained excitatory effects from NRM were observed in 35% of convergent neurons after 5-HT depletion by p-CPA. Davies and Roberts (1981)

showed that responses of dorsal horn neurons to iontophoretic application of SP were attenuated in a specific manner by iontophoretic application of 5-HT. In experiments done in our laboratory it was found that the excitability of all categories of spinal neurons to SP increased in 5-HT depleted cats (Jeftinija and Randić, 1980).

Norepinephrine A high density of norepinephrine-containing nerve terminals of the lower brain stem origin is present in the superficial laminae of the mammalian spinal dorsal horn (Carlsson et al., 1964; Fuxe, 1965; Nygren and Olson, 1977; Satoh et al., 1977; Pointras and Parent, 1978), the area where unmyelinated primary afferent fibers are shown to terminate (Light and Perl, 1979; Ralston and Ralston, 1979). K^+-evoked release of NE in vivo from the rat and cat spinal cord has been shown (Yaksh and Tyce, 1980). The iontophoretic administration of NE exerts inhibitory influence on the dorsal horn interneurons (Engberg and Ryall, 1966; Satoh et al., 1977; Belcher et al., 1978; Headley et al., 1978). This spinal action of NE has been frequently associated with descending inhibitory effects on spinal pain-transmission neurons produced by stimulation in the brain stem areas known to contain catecholamine-containing perikarya (Segal and Sandberg, 1977; Belcher et al., 1978; Akaike et al., 1978; Basbaum and Fields, 1979; Satoh et al., 1980; Hodge et al., 1981). In addition, a more general tonic brain stem inhibition of C-fiber inputs to dorsal horn interneurons has been reported (Handwerker et al., 1975; Soja and Sinclair, 1981). The precise neuronal mechanism of these descending inhibitions of spinal dorsal horn neurons is at present unknown. Although iontophoretically applied NE is shown to inhibit dorsal horn interneurons, it is not clear whether the effect is

mediated presynaptically or postsynaptically or both. Possible evidence for the presynaptic action of NE is the observation made by Phillis and Kirkpatrick (1979a) that catecholamines depolarize dorsal root terminals in the isolated toad spinal cord. Similarly, Apkarian et al. (1981) have shown that locus coeruleus stimulation causes NE-dependent primary afferent depolarization, which appears to be mediated by α-adrenergic receptors. At the spinal level involvement of α-adrenergic receptors has also been suggested by the findings that α-adrenergic agonists (NE and phenylephrine), but not β-adrenergic agonist (isoproterenol) significantly elevated the nociceptive threshold to thermal stimuli in unanesthetized animals (Reddy and Yaksh, 1980), and that systemically administered clonidine (an α_2-adrenergic agonist) inhibits the tail-flick reflex in spinalized rats (Kawasaki et al., 1978) and mice (Spaulding et al., 1979). Furthermore, the antinociceptive effect of NE was antagonized by α-adrenergic antagonists (phentolamine, phenoxybenzamine, tolazoline and yohimbine) but not affected by propranolol, a β-adrenergic antagonist (Reddy and Yaksh, 1980; Reddy et al., 1980). In addition, intrathecal administration of α-adrenergic antagonists partially antagonizes the analgesia produced by microinjections of morphine into the periaqueductal gray (Yaksh, 1979) or nucleus reticularis gigantocellularis (Kuraishi et al., 1979b). However, in experiments where single neuron activity was monitored NE-induced effects were not antagonized by α-adrenergic antagonists (Belcher et al., 1978). Similarly, we were not able to demonstrate any significant effect of α-adrenergic antagonists on NE-induced decrease in excitability of single cutaneous primary afferent fibers (Jeftinija et al., 1981b,c, 1982b).

Rationale

The background material reviewed indicates that the peptides cholecystokinin (CCK), somatostatin (SS), substance P (SP) and vaso-active intestinal polypeptide (VIP) as well as L-glutamate, 5-hydroxy-tryptamine (5-HT) and norepinephrine (NE) may function as neurotrans-mitters or neuromodulators in synaptic transmission processes in the central nervous system. As shown by neurochemical techniques high concentrations of these substances are present in the superficial parts of the spinal dorsal horn, the same area where primary afferent fibers of fine diameter (Aδ and C) are known to terminate. However, the functional role of these compounds in transmission and modulation of sensory input incoming into the spinal cord is far from being clear. The extent of our knowledge for some of them (CCK and VIP) is that they are present in the spinal dorsal horn. For others (SS, NE and L-glutamate) it is not clear whether they act presynaptically or postsynaptically or both. It was of interest therefore, to study the possible effects that CCK and VIP might have on the excitability of functionally identified cat spinal dorsal horn neurons. Since immunohistochemical and biochemical studies suggested a coexistence of 5-HT and SP in nerve terminals in the rat spinal cord, we felt that it would be useful to test the chemical sensitivity of cat spinal cord neurons to iontophoretically applied SP and to correlate the observed effects with changes in the spinal levels of 5-HT and SP. To test more directly for possible presynaptic action of NE, SS and L-glutamate, we have measured excitability changes of intra-spinal single cutaneous primary afferent C- and A-fibers during their application into the spinal cord. We hoped that this study would provide

new information necessary for our understanding of the role of the spinal dorsal horn in the transmission and modulation of sensory information coming into the spinal cord.

SECTION I. CHOLECYSTOKININ OCTAPEPTIDE EXCITES DORSAL HORN NEURONS BOTH *IN VIVO* AND *IN VITRO*[1,2]

Cholecystkinin (CCK), first purified from gut extracts as a peptide composed of 33 amino acids (Mutt and Jorpes, 1971), was later shown to be present in central and peripheral nerves (Dockray et al., 1978; Larsson and Rehfeld, 1979; Rehfeld et al., 1979). Both in the gut and in the brain CCK was found to consist of 5 main components of which the C-terminal octa- (CCK-8) and tetrapeptides (CCK-4) were the most abundant (Dockray et al., 1978; Rehfeld, 1978).

Within the central nervous system CCK is unevenly distributed (Rehfeld, 1978). In the guinea pig spinal cord, CCK-immunoreactive fibers form dense "caps" around the dorsal gray matter with small bundles of the immunoreactive fibers penetrating for short distances into the dorsal horns (Larsson and Rehfeld, 1979). Within neurons, CCK is concentrated in terminals where it apparently occurs in the synaptosomal vesicular fraction (Pinget et al., 1978; Rehfeld et al., 1979). Furthermore, in brain regions high in CCK content, the peptide binds specifically and reversibly to high affinity, saturable, receptor sites (Saito et al., 1980), and the K^+-induced, Ca^{2+}-dependent release of CCK from brain slices has been demonstrated (Rehfeld et al., 1979). These data suggest a possible role for CCK in synaptic transmission although functional features of brain systems that might use CCK as a neurotransmitter or

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[1]Published as a research paper by Jeftinija, S., V. Miletić, and M. Randić. 1981. Brain Res. 213:231-236. Elsevier/North-Holland Biomedical Press.

[2]*In vitro* experiments were performed by V. Miletić.

modulator are at present unknown. Applied by iontophoresis onto pyramidal cells in the rat hippocampal slice preparation in vitro, the peptide produces a strong and relatively rapid excitation accompanied by a depolarization of the neuronal membrane (Dodd and Kelly, 1979).

The superficial region of the dorsal horn of the mammalian spinal cord (laminae I-III) probably plays a major role in nociception as well as other sensory mechanisms (Christensen and Perl, 1970; Cervero et al., 1976). In addition, besides CCK, this region contains relatively high concentrations of opiate receptors (Atweh and Kuhar, 1977) and several other physiologically active peptides, such as substance P, somatostatin, enkephalin and neurotensin (Hökfelt et al., 1976; Snyder et al., 1978b), which have been shown to modify the excitability of the dorsal horn neurons in laminae I-III (Randić and Miletić, 1977, 1978; Miletić and Randić, 1979). It was of interest therefore, to study the central effects of CCK by applying the peptide onto functionally characterized dorsal horn units in the intact cat spinal cord. Since we have recently successfully utilized the rat spinal cord slice preparation (Takahashi, 1978) to study effects of the various peptides present in the dorsal horn (Miletić and Randić, 1980a) it was of additional interest to examine possible effects of CCK on the excitability of the dorsal horn neurons in vitro. In this paper, it will be shown that synthetic CCK octapeptide (CCK-8) produces a moderate to strong excitation of about half of all tested neurons in laminae I-VII, both in vivo and in vitro. This action of CCK appears to be a direct one exerted on postsynaptic sites of the dorsal horn units since it persists when synaptic transmission is blocked by superfusing the spinal cord

slices with a Ca^{2+}-free, Mg^{2+}-high Krebs solution. Preliminary results of our findings have been communicated (Jeftinija et al., 1980).

Experiments on intact spinal cord were performed on 8 adult cats (2-4 kg) initially anesthetized by halothane. The brain was anaemically destroyed by bilateral occlusion of the common carotid and vertebral arteries. The spinal cord was transected at the first cervical level. Thereafter, the animal was artificially respired and immobilized by succinylcholine chloride. Body temperature, blood pressure and end-tidal CO_2 concentration were monitored and maintained at optimal levels throughout the experiment. A laminectomy was made at the sacral and caudal spinal levels. High intensity mechanical stimuli were delivered to the skin of the tail (e.g. pressure from sharply pointed objects, grasping a fold of skin with calibrated forceps; when the latter type of mechanical stimulus was used on human skin, it provoked a highly painful sensation). Noxious radiant heat (skin heating to 45-55^{o}C) and low intensity mechanical stimuli (touching by resting a finger lightly on the skin, brushing with a camel's-hair brush) were also used. The activity of the dorsal horn neurons in laminae I-VII, activated by noxious mechanical and/or thermal stimulation or by an input in sensitive mechanoreceptors, was recorded through the central barrel of a "parallel" multibarrelled (2- or 3-barrelled) glass micropipette filled with a solution of Fast Green dye in 3M sodium chloride. The site of recording was marked by iontophoresis of the dye. The tip of the recording electrode protruded by about 5-20μm beyond the multibarrelled drug-containing assembly. Using electrodes of this type both the size and the stability of recorded units were greatly improved. Conventional microiontophoretic

technique was used to study the effect of synthetic CCK-8 (4.0 mM in 165 mM NaCl, pH 7.8, Boehringer-Mannheim, ejected as an anion) on the spontaneous firing of the dorsal horn neurons. In all experiments, an adjacent barrel of the micropipette drug assembly routinely contained a solution of 165 mM NaCl at a similar pH as CCK in order to eliminate any possible current or pH artifacts, and to provide a current return path while ejecting CCK. The iontophoretic unit used contained a current balancing circuit.

For the in vitro experiments, Sprague-Dawley rats (2-9 days old) were anesthetized with ether and, following a laminectomy, a 1-2 cm segment of lumbo-sacral spinal cord, with about 0.5 cm of attached dorsal rootlets, was excised. The spinal cord was then quickly cut into transverse 300-800μm thick slices, by hand with a razor blade or with a tissue chopper, and placed on a fine nylon mesh in the recording chamber. There the slices were continuously perfused with warm ($32\pm1^{\circ}C$), aerated (95% O_2 and 5% CO_2) Krebs solution (105 mM NaCl, 4.7 mM KCl, 2.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.7 mM D-glucose, pH 7.4) at a rate of about 3 ml/minute. Following an incubation period of 45-60 min recording of single unit activity commenced since the dorsal horn neurons appeared physiologically intact exhibiting conventional resting, synaptic and action potentials. Spontaneous and/or evoked (electrical stimulation of the dorsal rootlet stump with a glass-insulated Pt-electrode) single unit activity was recorded with a 'parallel' multibarrelled microelectrode as described above. CCK-8 was applied only by iontophoresis.

The results of our experiments are based on data obtained from 49

units. Of these 40 were studied in the intact cat spinal cord (Table 1), and 9 in the rat spinal cord slices, *in vitro*. The units recorded in the intact cat spinal cord were classified into the 3 classes of neurons recognized in spinal preparations of cats in this area on the basis of their excitability by different kinds of cutaneous afferent input (Cervero et al., 1976)(Table 1). Twelve were of the class 3 type (excited only by noxious mechanical and/or thermal stimuli), 11 of the class 2 type (activated by nociceptors as well as sensitive mechanoreceptors), and 17 of the class 1 type (excited by an input in sensitive mechanoreceptors only). All of the histologically recovered recording sites (n=12) were found no deeper than lamina VII (seven neurons were recorded in laminae I-IV, five in laminae V-VII).

Table 1. Summary of responses of cat dorsal horn neurons (laminae I-VII) to cholecystokinin

Class	Total	Excited	No effect
1	17	9	8
2	11	5	6
3	12	5	7
	40	19	21

Class 1: activated by sensitive mechanoreceptors only.
Class 2: activated by sensitive mechanoreceptors and nociceptors.
Class 3: activated by nociceptors only.

When CCK was applied as an anion with currents of 30-200 nA for periods up to 3 min, a moderate to strong excitation (20-120% above control levels) of about half (19/40) of the tested units in the intact cat spinal cord was seen (Table 1). Excitation was observed as initiation

of firing in a previously quiescent unit, or as an increase in the rate of spontaneous firing (Figs. 1 and 2). Generally, the excitant response to CCK was of relatively slow onset and recovery, although some fast responses were also seen. The peptide proved to possess an excitatory action in all categories of neurons earlier described (Cervero et al., 1976). Thus, unlike substance P, somatostatin and methionine-enkephalin, but similarly to neurotensin, CCK showed no specificity in its action towards the nociceptive population of neurons located in laminae I-III (Randić and Miletić, 1977, 1978; Miletić and Randić, 1979).

A typical excitatory response to CCK of a unit recorded in the intact cat spinal cord, and activated by both nociceptors and sensitive mechanoreceptors (class 2), is illustrated in Fig. 1. A continuous application of CCK (150 nA) for about 2.5 min produced a 40% increase (above control levels) in the spontaneous firing rate of this unit. Negative current of the same magnitude (150 nA), applied through the NaCl barrel, was without effect. Upon histological examination of the recording site, as shown in the insert of Fig. 1, we found the neuron located at the border zone between laminae III and IV. Another example of the excitatory response to CCK of a class 2 unit recorded in the intact cat spinal cord is illustrated in Fig. 2. A continuous application of CCK (120 nA) for more than 3 min produced an instantaneous increase in the spontaneous firing rate of this unit. This excitation outlasted the period of application for about 4 min. Negative current (120 nA) applied through the NaCl barrel, had no similar effect. Histological examination revealed this neuron to be located in lamina V (Fig. 2 insert).

A strong excitation, with a somewhat faster onset but still relatively

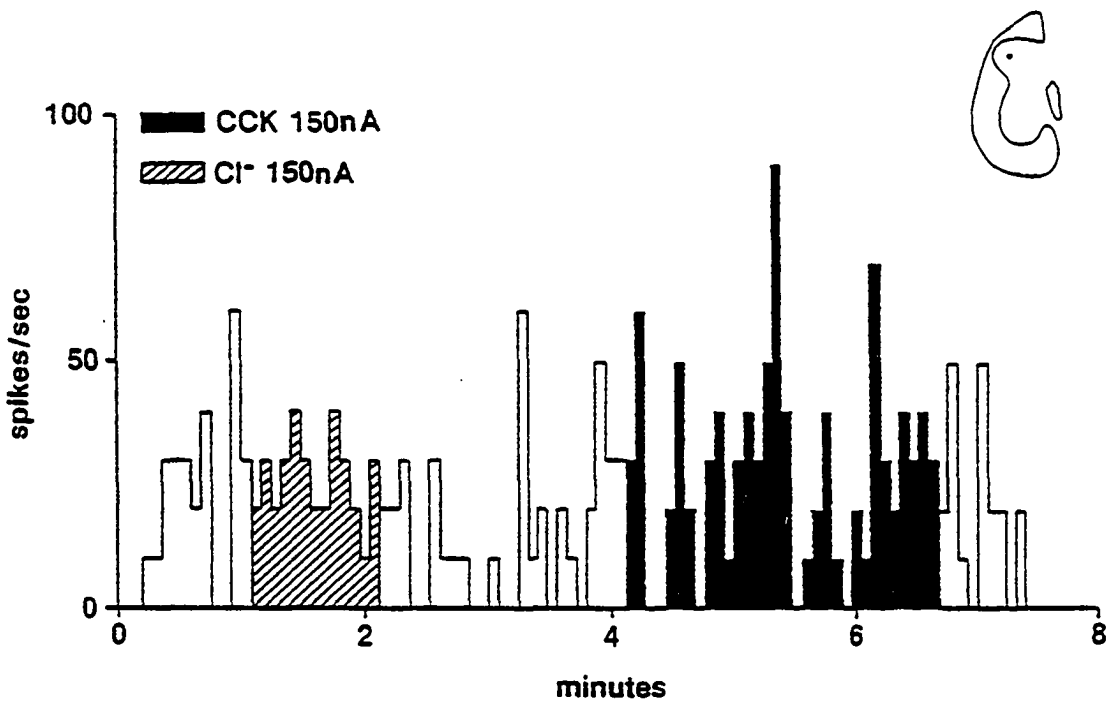

Figure 1. Excitation of a spontaneously active unit in the intact cat spinal cord produced by iontophoretic application of CCK (150 nA). Negative current, applied through the NaCl barrel, (150 nA), was without effect. The neuron, located in the border zone between laminae III and IV, was activated by both nociceptors and sensitive mechanoreceptors (class 2). Unit activity was recorded on moving film and the discharge frequency counted

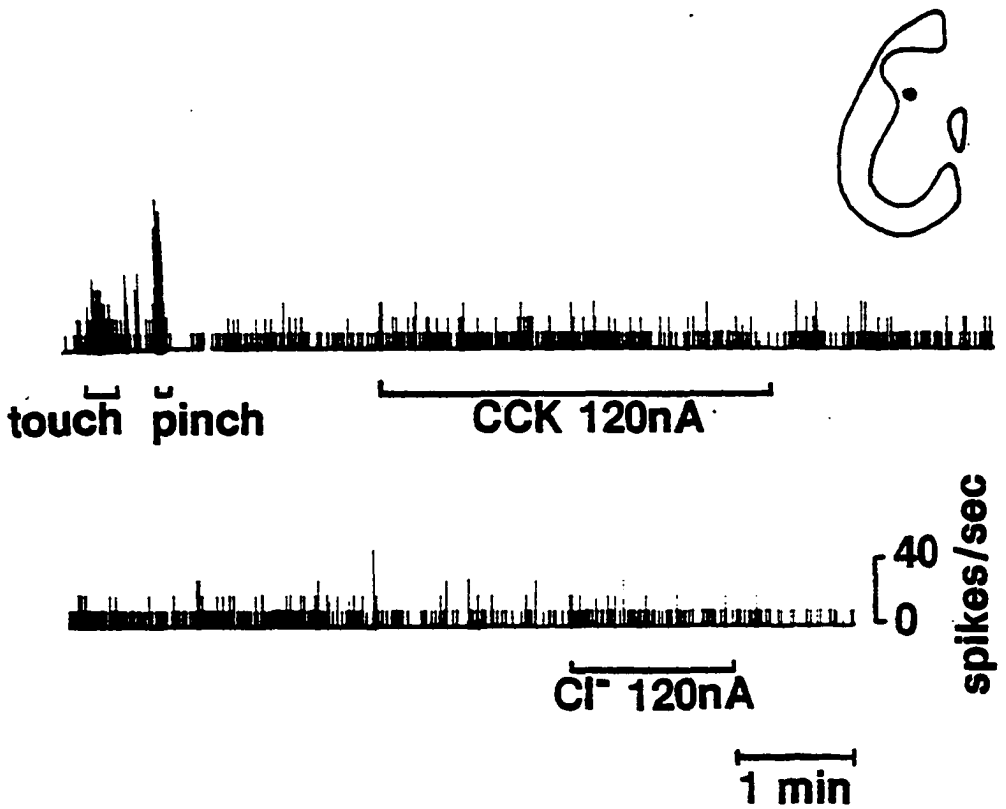

Figure 2. Iontophoretic application of CCK (120 nA) for about 3 min produced an excitation which outlasted the period of application by some 4 min. Negative current (120 nA), applied through the NaCl barrel, had no similar effect. The cell was activated by an input in both nociceptors and sensitive mechanoreceptors (class 2) and was located in lamina V. Rate-meter record of a spontaneously active unit in the intact cat spinal cord

slow recovery, was also seen in 5 of 9 tested units recorded in vitro from slices of rat spinal cord. All of the 9 units were located superficially to lamina VI, as judged by observing the recording site under a stereomicroscope at appropriate magnification (50X). As illustrated in Fig. 3, CCK (150 nA) initiated firing of a previously quiescent dorsal horn neuron with a latency of about 7 sec. Again, excitation outlasted the period of peptide application by some 30 sec. Negative current (150 nA), applied through the NaCl barrel, was without effect. The excitatory response to CCK was not altered when the superfusing solution ('normal' Krebs) was replaced with a Ca^{2+}-free, Mg^{2+}-high bathing medium (0 mM Ca^{2+}, 8 mM Mg^{2+}) which solution blocked synaptic transmission as evidenced by the fact that the dorsal rootlet stimulation was ineffective in evoking activity in the dorsal horn. The latter finding indicates that the action of CCK-8 might be a direct one exerted on the postsynaptic sites of dorsal horn units.

In summary then, we have shown that: 1) both in vivo and in vitro CCK, applied iontophoretically, causes a moderate to strong excitation of about half of all tested neurons in laminae I-VII of the spinal cord, 2) the excitation is observed in all categories of units recognized in spinal preparations of cats in this area on the basis of their excitability by different kinds of cutaneous afferent input, and 3) CCK action appears to be a direct one exerted on postsynaptic sites of the dorsal horn neurons.

The characteristics of the CCK-produced excitation and the results obtained in the in vitro preparation are consistent with the possibility that CCK acts on postsynaptic sites in the dorsal horn of the mammalian

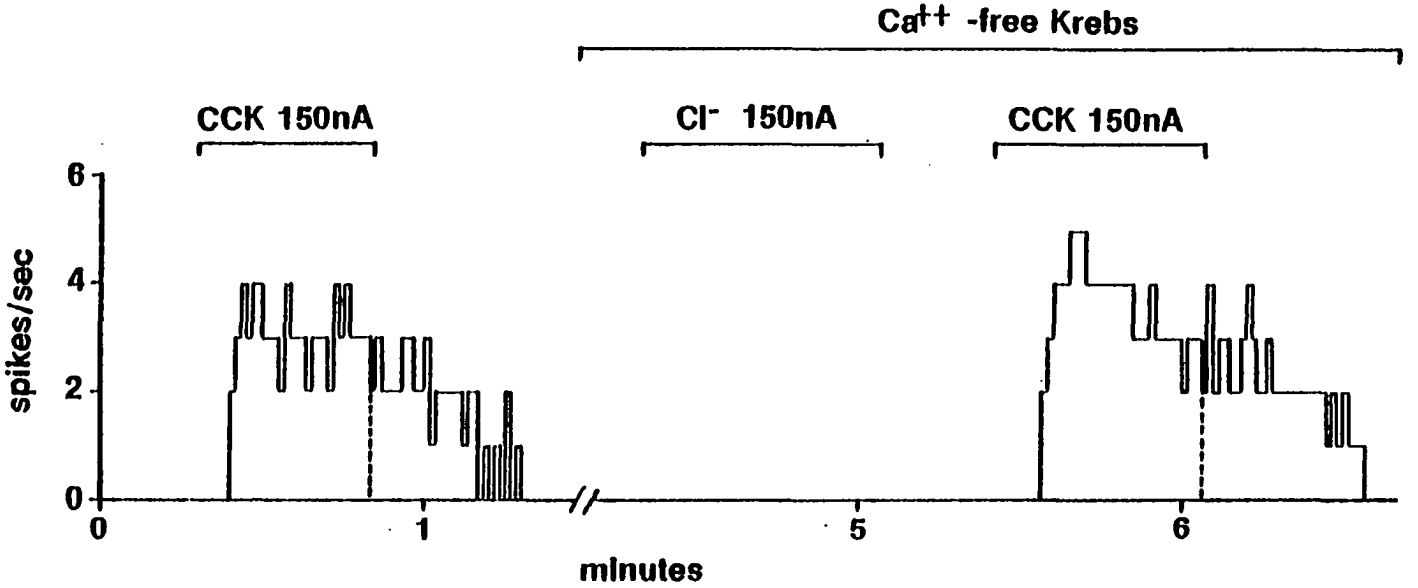

Figure 3. Excitation of a superficial dorsal horn neuron in the rat spinal cord slice preparation by iontophoretically applied CCK (150 nA). The excitation is seen to persist in a Ca^{++}-free, Mg^{++}-high superfusing solution. Negative current (150 nA) is without effect

spinal cord as a neurotransmitter or modulator.

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SECTION II. VASOACTIVE INTESTINAL POLYPEPTIDE EXCITES MAMMALIAN DORSAL HORN NEURONS BOTH *IN VIVO* AND *IN VITRO*[1,2]

Vasoactive intestinal polypeptide (VIP), a 28 amino acid residue peptide, originally isolated from porcine duodenum (Said and Mutt, 1970a; Mutt and Said, 1974) was later shown to be present in the nervous system as well (Bryant et al., 1976; Larsson et al., 1976b; Said and Rosenberg, 1976; Lorén et al., 1979). Cytochemical and radioimmunochemical studies have revealed the presence of numerous VIP-positive nerve cell bodies and terminals in the mammalian brain (Larsson et al., 1976a,b; Said and Rosenberg, 1976; Fuxe et al., 1977; Besson et al., 1979). In the rat spinal dorsal horn, thin VIP-immunoreactive fibers with large varicosities, probably of dorsal root ganglia origin (Hökfelt et al., 1980; Yaksh et al., 1982), are present in substantial numbers in laminae I and II. In addition, fibers containing VIP are seen around the central canal and a few in lamina VII (Gibson et al., 1981). VIP-like immunoreactivity has also been detected in the cat spinal dorsal horn (Yaksh et al., 1982). Within neurons VIP is concentrated in terminal-like varicosities, where it apparently occurs in the synaptosomal vesicular compartment (Giachetti et al., 1977; Emson et al., 1978). Ca^{2+}-dependent release of VIP from the brain *in vitro* preparations has been demonstrated (Giachetti et al., 1977; Emson et al., 1978). In addition, the electrical stimulation of the cat sciatic nerve evoked release of VIP, only when stimulus parameters were of

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[1]This manuscript is submitted for publication as a research paper by S. Jeftinija, K. Murase, V. Nedeljkov and M. Randić to Brain Research.

[2]*In vitro* experiments were performed by K. Murase and V. Nedeljkov.

such intensities to activate small diameter primary afferent fibers (Yaksh et al., 1982). The binding of radiolabeled VIP to a site on rat brain membranes was demonstrated (Taylor and Pert, 1979). VIP excites spontaneously active cerebral cortical neurons in rats and depolarizes motoneurons in the toad spinal cord (Phillis et al., 1978). Applied by pressure microinjection onto pyramidal neurons in the CA1 region of the rat hippocampal slice, VIP produced a rapid and potent excitation accompanied by a depolarization of the neuronal membrane and a large fall in membrane input resistance (Dodd et al., 1979).

The superficial region of the dorsal horn of the mammalian spinal cord (laminae I-III) probably plays a major role in nociception as well as other sensory mechanisms (Cervero and Iggo, 1980). In addition, besides VIP this region contains relatively high levels of several physiologically active peptides, such as substance P, somatostatin, enkephalin, neurotensin and cholecystokinin (Hökfelt et al., 1976; Snyder et al., 1978b; Gibson et al., 1981), which have been shown to modify the excitability of the dorsal horn neurons (Randić and Miletić, 1977, 1978; Miletić and Randić, 1979; Jeftinija et al., 1981a). It was of interest, therefore, to study the central effects of VIP by applying the peptide locally onto functionally characterized dorsal horn units in the intact cat spinal cord. In addition, responses of dorsal horn neurons to bath application of VIP were studied by intracellular recording in the rat spinal cord slice preparation. In this paper, it will be shown that VIP produces a strong excitation of more than 75% of all tested neurons in laminae I-VII, both <u>in vivo</u> and <u>in vitro</u>. Preliminary results of our findings have already been communicated (Randić and Jeftinija, 1981).

Experiments on intact spinal cord were performed on 17 adult cats (2-4 kg) initially anesthetized by halothane. The brain was anaemically destroyed by bilateral occlusion of the common carotid and vertebral arteries. The spinal cord was transected at the first cervical level. Thereafter, the animal was artificially respired and immobilized by constant infusion of succinylcholine chloride. Body temperature, blood pressure and end-tidal CO_2 concentration were monitored and maintained at optimal levels throughout the experiment. The spinal cord was exposed by laminectomy at the lumbosacral and caudal spinal levels. The exposed cord was covered with 2-4% agar dissolved in lactated Ringer's solution (Hartmann's solution, Abbott Labs). To minimize movements of the spinal cord associated with respiration, bilateral pneumothorax was made as a routine. In some experiments, a sciatic nerve or a coccygeal dorsal root (or rootlet) was freed from surrounding tissue and placed on a bipolar stimulating electrode distally and a bipolar recording electrode centrally, leaving both peripheral and central connections intact. This electrode arrangement served for electrical stimulation of the sciatic nerve or the dorsal root and recording of compound action potentials, of different kinds of myelinated and unmyelinated afferent fibers. The electrical stimulus thus acted as a search stimulus for units activated by afferent volleys conducted in Aδ- or C-fiber groups. High intensity mechanical stimuli were delivered to the skin of the tail or left hindlimb (e.g. pressure from sharply pointed objects, grasping a fold of skin with calibrated forceps; when the latter type of mechanical stimulus was used on human skin it provoked a highly painful sensation). Low intensity mechanical stimuli (touching by resting a finger lightly on the skin,

brushing the skin with a camel's-hair brush) were also used. The activity of the dorsal horn neurons in laminae I-VII, activated by noxious mechanical stimulation or by an input in sensitive mechanoreceptors, was recorded through the central barrel of a "parallel" multibarrelled (3- or 4-barrelled) glass micropipette filled with a solution of Fast Green dye in 3 M sodium chloride. The site of recording was marked by iontophoresis of the dye. The tip of the recording electrode protruded by about 15-20 μm beyond the multibarrelled drug-containing assembly. Using electrodes of this type, both the size and the stability of recorded units were greatly improved. Conventional microiontophoretic or pressure microinjection techniques were used to study the effect of synthetic VIP (0.7-1.4 mM in 20 mM acetic acid, pH 4.5-5.3, Peninsula, ejected as cation) on the spontaneous or evoked firing of the functionally identified spinal dorsal horn neurons. In all experiments, an adjacent barrel of the micropipette drug assembly routinely contained a solution of 20 mM sodium acetate at a similar pH as VIP in order to eliminate any possible current or pH artifacts, and to provide a current return path while ejecting VIP. The iontophoretic unit used contained a current balancing circuit.

Experiments in vitro were performed on 8-19 days old Sprague-Dawley rats. The animals were anesthetized with ether, and a laminectomy performed to expose the lower-thoracic and lumbosacral spinal cord together with dorsal roots. Following laminectomy, about 1-1.5 cm long segment of lumbosacral spinal cord, with attached dorsal rootlets was quickly excised and immersed into aerated (95% O_2 and 5% CO_2) Krebs-Ringer solution at 37°C. The composition of the solution was (mM): NaCl 124; KCl 5; KH_2PO_4 1.2; $CaCl_2$ 2.4; $MgSO_4$ 1.3; $NaHCO_3$ 26; glucose 10, pH 7.4. After

the removal of the pia mater on the lateral aspects of the spinal cord, the spinal segment was cut manually into 5 mm blocks, and one of the blocks of tissue affixed with cyanoacrylic glue (Borden, Inc.) to the bottom of a Plexiglass cutting chamber of an Oxford Vibratome. The spinal cord block was positioned in the bath of the Vibratome with the dorsal surface of the spinal cord facing the vibrating blade. The bath of the Vibratome had previously been filled with the Krebs solution, maintained at 37°C and bubbled with a mixture of oxygen and carbon dioxide. The blade of the Vibratome was positioned 300 μm below the dorsal surface of the spinal cord, and the spinal segment sectioned to yield two horizontal 300 μm-thick dorsal horn slices. The duration of the entire procedure from the removal of the spinal cord until the slice was made rarely exceeded 5 min. In a few experiments, transverse slices were used. The slices were incubated in Krebs-Ringer solution at 37°C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Krebs-Ringer solution (NaCl 127 mM; KCl 1.9 mM; $CaCl_2$ 2.4 mM; $MgSO_4$ 1.3 mM; $NaHCO_3$ 26 mM; glucose 10 mM, occasionally containing 0.001% H_2O_2) at 33 ± 1°C at a flow rate of about 1 ml/min. The recording chamber had a capacity of 0.5 ml. Intracellular recordings were performed with micropipettes filled with 3 M K-acetate having D.C. resistances of 60-100 MΩ. A high-input impedance bridge amplifier (WP Instruments, M707) was used to inject current through the recording microelectrode. The amplitude of the recorded voltage produced by rectangular hyperpolarizing current pulses (0.1-0.3 nA, 50 msec duration applied at 0.1-0.2 Hz) was used as a measure of the membrane input resistance. VIP was applied by bath perfusion in concentrations of

10^{-8}-10^{-5} M.

The results of our experiments are based on data obtained from 80 units. Of these, 61 were studied in the intact cat spinal cord (Table 1), and 19 in the rat spinal cord slices, in vitro. The units recorded in the intact cat spinal cord were classified into the 3 categories of neurons recognized in spinal preparations of cats in this area on the basis of their excitability by different kinds of cutaneous afferent input. 22 were of the class 1 type (excited by an input in sensitive mechanoreceptors only), 20 of the class 2 type (activated by nociceptors as well as sensitive mechanoreceptors), and 19 of the class 3 type (excited only by noxious mechanical and/or thermal stimuli) (Table 1). Majority of the histologically recovered recording sites (n=25) were found no deeper than lamina VII (Fig. 1).

Table 1. Summary of responses of cat dorsal horn neurons (laminae I-VII) to vasoactive intestinal polypeptide

Class	Total	Excited	No effect
1	22	16	6
2	20	16	4
3	19	14	5
	61	46	15

Class 1: activated by sensitive mechanoreceptors only.
Class 2: activated by sensitive mechanoreceptors and nociceptors.
Class 3: activated by nociceptors only.

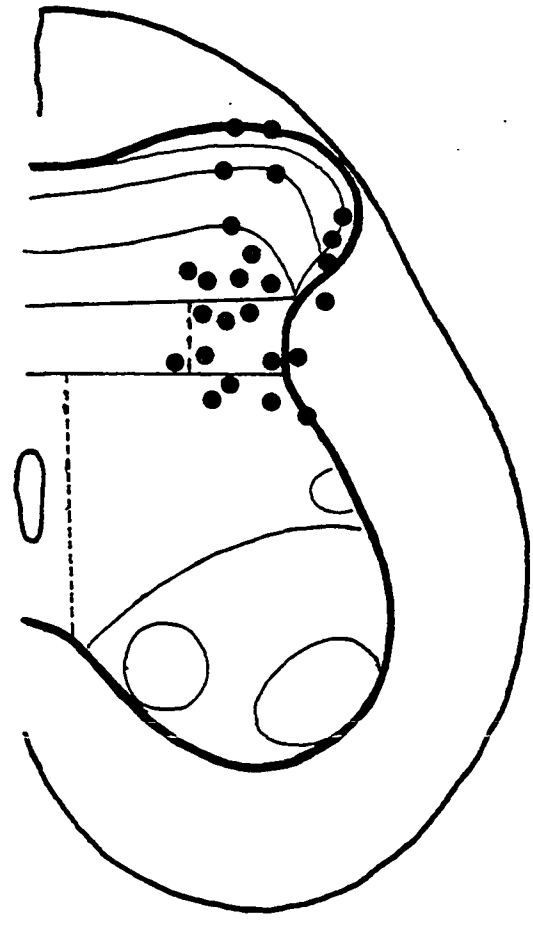

Figure 1. Schematic reconstruction of the location of neurons excited by VIP from which extracellular recordings were made.

When VIP was applied as cation with currents ranging from 25 to 100 nA or with an average positive pressure of 5 psi for periods up to 3 min, it proved to be a potent excitant of about 75% of dorsal horn neurons tested. The remainder were unaffected (Table 1). Excitation was observed as initiation of firing in a previously quiescent cell (Fig. 2C) or as an increase in the rate of spontaneous firing (Figs. 2A,B). The latency of the excitation was variable ranging from 2-60 sec in the class 1 type units (38.4 ± 6.2, mean ± S.E.M.), and being somewhat longer in the class 2 and 3 type units (71.4 ± 7.6). The excitant action of VIP often continued for several minutes after its application was terminated. Although the effective concentrations of VIP varied significantly from cell to cell, on a given neuron the degree of excitation was related to the dose of VIP applied. VIP proved to possess an excitatory action in all categories of neurons earlier described (Cervero et al., 1976). Thus, unlike substance P, somatostatin and met-enkephalin, but similarly to neurotensin and cholecystokinin, VIP showed no specificity in its action towards the nociceptive population of neurons located in laminae I-III (Randić and Miletić, 1977, 1978; Miletić and Randić, 1979; Jeftinija et al., 1981a). Repeated applications of VIP at intervals less than 5 min led to progressively smaller responses.

Typical excitatory responses to VIP of 3 units recorded in the intact cat spinal cord, and activated either by sensitive mechanoreceptors (Fig. 2A), or by both nociceptors and sensitive mechanoreceptors (Fig. 2B), or only by nociceptors (Fig. 2C) are illustrated in Fig. 2. As seen in Fig. 2A, a continuous application of VIP (50 nA) for about 2.5 min produced marked increase in the spontaneous firing of this class 1 unit within

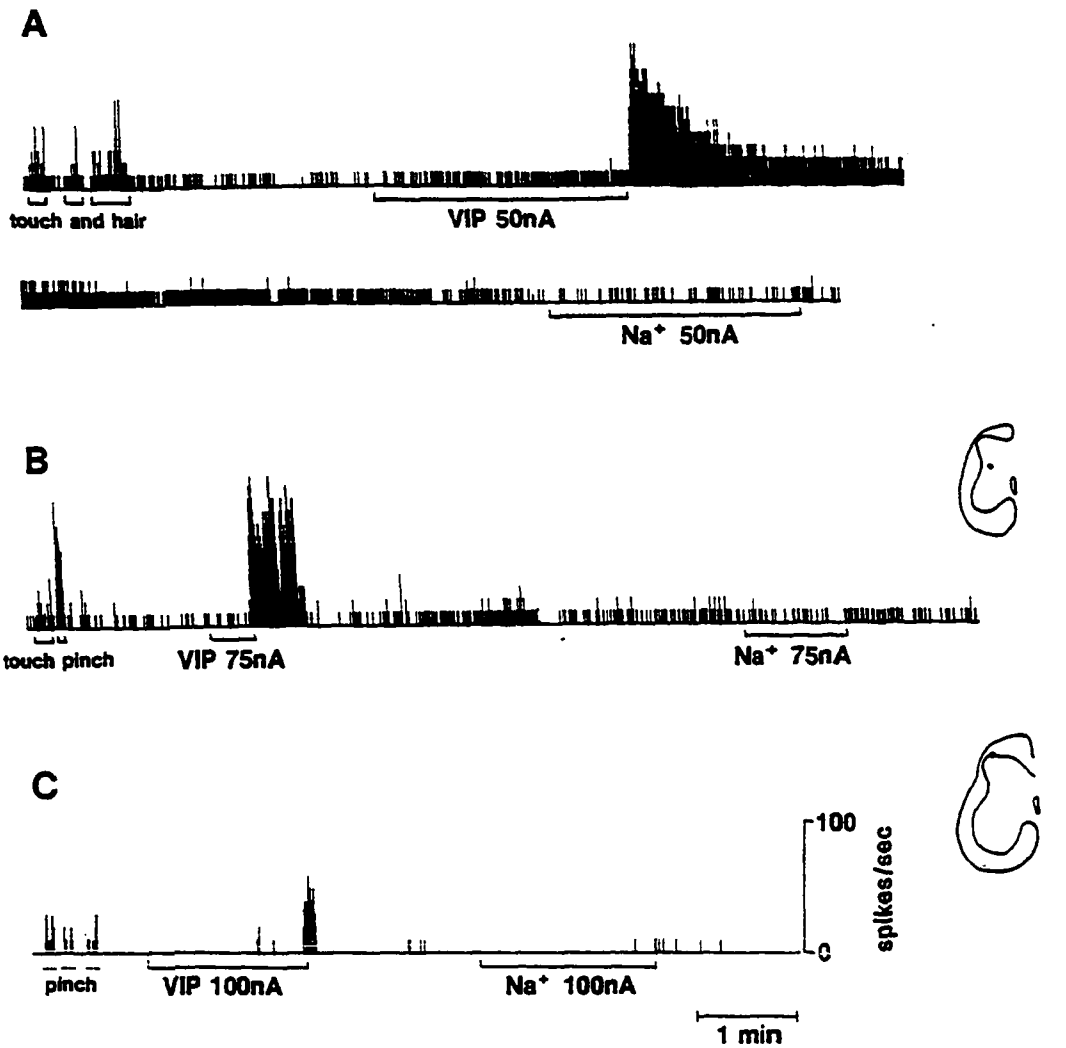

Figure 2. Typical excitatory responses to VIP of 3 units recorded in the intact cat spinal cord, and activated by sensitive mechanoreceptors (A), or by both nociceptors and sensitive mechanoreceptors (B), or by an input in nociceptors only (C). Positive current applied through the sodium acetate barrel, had no similar effect. Rate-meter records of unitary activity in the intact cat spinal cord. Location of neurons from which extracellular recordings were made shown in the insert of Figs. 2B,C

12 sec following the onset of VIP current. On turning off the current expelling VIP, the excitation persisted for about 7 min. Positive current of the same magnitude (50 nA), applied through the NaCl barrel, was without effect. Another example of the excitatory response to VIP of a class 2 unit is illustrated in Fig. 2B. Here a continuous application of VIP (75 nA) for 25 sec caused excitation within 22 sec and an after-discharge lasting about 30 sec. Histological examination revealed the neuron to be located in lamina V (Fig. 2B insert). The unit illustrated in Fig. 2C did not show any background activity, however, it was activated by noxious mechanical stimulation of the skin. A continuous application of VIP (100 nA) for about 95 sec caused excitation within 90 sec and a relatively brief afterdischarge. On histological examination of the recording site, we found that the unit was located in the marginal cell layer (Rexed's lamina I) as illustrated in the insert of Fig. 2C.

Dorsal horn neurons were impaled up to 12 h after the slice was prepared. Stable intracellular recordings were made from single neurons for as long as 5 h. During the period of 5-10 min before VIP application, control responses to constant current hyperpolarizing pulses and direct intracellular stimulation were monitored. Only cells which showed uniform responses and stable membrane potential during this period were used in further analysis. Mean action potential amplitude and membrane potential were 65 mV ($\pm$ 10, S.D.) and -66 mV ($\pm$ 10), respectively. In 18 out of 19 tested cells, application of VIP into the bathing medium (10^{-8} to 7×10^{-6}M for 1 min) produced depolarization that is slow both in onset and recovery (Fig. 3). On several occasions, the depolarization was of sufficient strength to cause inactivation of the spike generating mechanism. VIP-

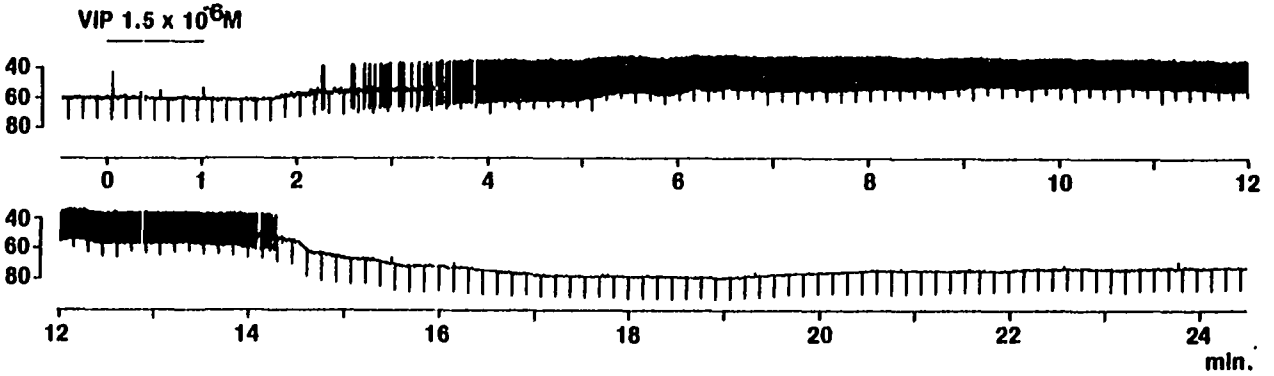

Figure 3. The excitatory effect of vasoactive intestinal polypeptide (VIP) on a dorsal horn neuron recorded with an intracellular microelectrode in a spinal cord slice preparation of 12 day-old rat. Cell input resistance was measured by recording membrane responses to constant hyperpolarizing current pulses (0.2 nA, 50 ms). Bath application of VIP (1.5×10^{-6} M) for a min causes a depolarization that is slow in onset and recovery. VIP-induced depolarization and increased synaptic activity are in this cell accompanied by unusually prolonged firing of action potentials. The depolarization was followed by a marked hyperpolarizing response. A continuous record

induced depolarization is in many neurons accompanied by increased numbers of synaptic potentials, as evidenced in Fig. 3 with an increase in the baseline noise. In addition, the dorsal horn neurons frequently fired action potentials. Application of VIP (10^{-6} M) decreased the input resistance of 13 of 16 tested cells, and increased resistance in 1 cell. VIP-depolarization was occasionally followed by a marked hyperpolarizing response (Fig. 3).

The present pharmacological demonstration of a potent excitatory action of VIP on cat spinal dorsal horn neurons is complementary to the similar observations of Phillis et al. (1978) on rat cerebral cortical and frog spinal neurons; Salt and Hill (1981) on rat neurons of the nucleus caudalis of the trigeminal nuclear complex, Dodd et al. (1979) on CA1 neurons of the rat hippocampus, and Williams and North (1979) on neurons of the myenteric plexus. In addition, our results obtained in the neonatal rat spinal cord slice preparation confirm the observation made by Dodd et al. (1979) that VIP causes a dose-dependent depolarization, accompanied by a fall in membrane input resistance.

VIP has been shown by immunohistochemical methods to be present within some primary sensory neurons (Hökfelt et al., 1980) and VIP-positive fibers are concentrated in laminae I and II. In addition, smaller numbers of fibers containing VIP are seen around the central canal and in lamina VII (Gibson et al., 1981). VIP apparently occurs in the synaptosomal vesicular compartment and a Ca^{2+}-dependent release of VIP has been demonstrated from brain <u>in vitro</u> preparations (Giachetti et al., 1977; Emson et al., 1978). Sciatic nerve stimulation also released VIP, but only at stimulus intensities which activated Aδ- and C-fibers (Yaksh et al.,

1982). These data coupled with our findings of a potent excitatory action of VIP on spinal dorsal horn neurons suggest a possible physiological role for VIP in synaptic function, either as a transmitter or as a modulator of the actions of other transmitters.

This work was supported by USPHS Grant NS17297 and BNS23871.

SECTION III. INCREASED EXCITABILITY OF DORSAL HORN NEURONS TO SUBSTANCE P IN p-CHLOROPHENYLALANINE-PRETREATED CATS

The coexistence of several putative neurotransmitters in a single neuron has been demonstrated in invertebrates (Kerkut et al., 1976; Brownstein et al., 1974) and in the mammalian peripheral and central nervous systems (see Hökfelt et al., 1980). Recent reports suggest that 5-hydroxytryptamine (5-HT) and substance P (SP) are contained in the same neurons in various parts of the central nervous system especially in the region of the raphe nuclei and descending raphe-spinal pathways (Chan-Palay et al., 1978; Hökfelt et al., 1978; Björklund et al., 1979; Chan-Palay, 1979). Particularly functionally interesting is the possibility of a descending "5-HT + SP" projection to the dorsal horn, where both substances have been ascribed as a neurotransmitter and/or as a neuromodulator role.

The nucleus raphe magnus (NRM) has been found to be the major source of dorsal horn 5-HT (Dahlström and Fuxe, 1965; Oliveras et al., 1977), and iontophoretic application of 5-HT depresses the activity of dorsal horn neurons with noxious inputs (Randić and Yu, 1976; Belcher et al., 1978; Headley et al., 1978; Jordan et al., 1978). Electrical stimulation of the medullary raphe produces analgesia (Oliveras et al., 1975) and suppresses the activation of dorsal horn neurons by noxious peripheral stimuli (Fields et al., 1977; Guilbaud et al., 1977; Willis et al., 1977; Duggan and Griersmith, 1979). Descending inhibitory influences from NRM on responses of dorsal horn neurons to C-fiber inputs are reduced after 5-HT depletion by p-chlorophenylalanine (p-CPA). In addition, long-lasting and sustained excitatory effects from NRM were observed in 35% of convergent

neurons after 5-HT depletion by p-CPA (Rivot et al., 1980). These data have linked descending 5-HT raphe-spinal pathway with a control of the spinal transmission of nociceptive information.

SP, has been shown to exert an excitant action on nociceptive dorsal horn neurons (Henry, 1976; Randić and Miletić, 1976, 1977); this and other indirect evidence provided a support for the concept that SP is involved in synaptic transmission from primary afferents that mediate nociception.

We have presently pursued the question of close topographic and/or functional interdependence of 5-HT and SP in the cat spinal cord by examining whether the excitability of functionally identified dorsal horn neurons in response to iontophoretic application of SP is affected by p-CPA pretreatment. In this report, it will be shown that the excitability of dorsal horn neurons to substance P increases in p-CPA-pretreated cats. In addition, it will be shown that level of 5-HT was decreased to about 15% of control and that levels of SP, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK) and neurotensin (NT) were not convincingly changed in spinal cord of p-CPA-pretreated animals. Preliminary results of our findings have already been communicated (Jeftinija and Randić, 1980).

Electrophysiological experiments were performed on 40 adult cats (2-4 kg). Eight cats, out of 40, were pretreated with p-CPA (DL-p-chlorophenylalanine methyl ester, hydrochloride, Calbiochem-Behring Corp. 400 mg/kg i.p.) 3 days prior to testing. The drug was dissolved in saline warmed to 40^{o}C. On the day of experiment, cats were initially anesthetized by halothane. The brain was anemically destroyed by bilateral occlusion of the common carotid and vertebral arteries. The spinal cord was tran-

sected at the first cervical level. Surgical and recording procedures were identical to those already described in Sections I and II. The activity of the spinal cord neurons, activated by noxious mechanical stimulation, by an input in sensitive mechanoreceptors or by proprioceptors, was recorded through the central barrel of a "parallel" multibarrelled (2- or 4-barrelled) glass micropipette filled with a solution of Fast Green dye in 3 M sodium chloride. The site of recording electrode was marked by iontophoresis of the dye. The tip of the recording electrode protruded by about 10-20 μm beyond the multibarrelled drug-containing assembly. Using electrodes of this type, both the size and the stability of recorded units were greatly improved. Conventional iontophoretic technique was used to study the effect of SP (3.7 mM in 20 mM acetic acid, pH 5.5, Beckman, ejected as a cation) on the spontaneous firing of the dorsal horn neurons. In all experiments, an adjacent barrel of the micropipette drug assembly routinely contained a solution of 20 mM sodium acetate at a similar pH as SP in order to eliminate any possible current of pH artifacts. The iontophoretic unit used contained a current balancing circuit.

Additionally, 5 cats (2-4 kg) were pretreated with p-CPA (350 mg/kg i.p.) for neurochemical studies. To avoid lethal toxic effects sometimes seen in cats pretreated with 400 mg/kg of p-CPA, the dose of p-CPA in this group of experiments was reduced to 350 mg/kg. Three days after pretreatment, cats were anesthetized with fluothane and laminectomy was performed at the lumbosacral and caudal spinal levels. Segments of spinal cord from the seventh lumbar to the second or third caudal were taken out, frozen with liquid nitrogen, and stored in dry ice. The left half of the spinal

cord was used for analysis of peptide levels and the right half for analysis of 5-HT levels. Determination of 5-HT levels was performed by Dr. Alice Larson (Department of Veterinary Biology, University of Minnesota, St. Paul, Minn.) using a modification of the procedure described by Maruyama and Takemori (1971). Determination of peptide levels was performed by Dr. Tony Yaksh (Departments of Neurosurgery and Pharmacology, Mayo Clinic, Rochester, Minn.) using radioimmunoassay (Yaksh et al., 1980, 1982).

The results of our experiments are based on data obtained from 37 units from p-CPA-pretreated cats and 92 units from untreated cats (Table 1). The units recorded in the intact cat spinal cord were classified into four categories on the basis of their excitability by different kind of inputs. Forty-two in control cats and 15 in p-CPA-pretreated cats were of class 1 type (excited by an input in sensitive mechanoreceptors only), 40 in control and 16 in pretreated of the class 2 and 3 type (activated by nociceptors as well as sensitive mechanoreceptors or excited only by noxious mechanical and/or thermal stimuli), and 10 in control and 6 in pretreated cats were excited by proprioceptors. The majority of the histologically recovered recording sites (13 out of 16) in the spinal cord of p-CPA-pretreated cats were found no deeper than lamina VII (Fig. 1).

When SP was applied as a cation with currents of 20 to 100 nA for periods up to 3 min, a moderate to strong excitation of about 31% of the tested class 1 and 80% of class 2 and 3 of spinal cord neurons of untreated cats was seen (Table 1). In contrast to this finding, all but one unit irrespective of the type of their sensory input, were strongly excited by SP in the animals pretreated with p-CPA. Excitation was observed as initiation of firing in a previously quiescent unit, or as an increase in rate of spon-

Table 1. The effects of substance P (20-100 nA/up to 3 min) on functionally identified dorsal horn neurons. The figures represent the numbers of cells excited, depressed or unaffected. SP was tested on spontaneous activity

	Untreated cats				p-CPA-treated cats			
Input	Total number of cells	Excited	Depressed	No effect	Total number of cells	Excited	Depressed	No effect
Class 1	42	13	0	29	15	15	0	0
Class 2 and Class 3	40	32	0	8	16	16	0	0
Units activated by proprioceptors	10	5	1	4	6	5	1	0

Class 1: Units activated by sensitive mechanoreceptors only.
Class 2: Units activated by sensitive mechanoreceptors and nociceptors.
Class 3: Units activated by nociceptors only.

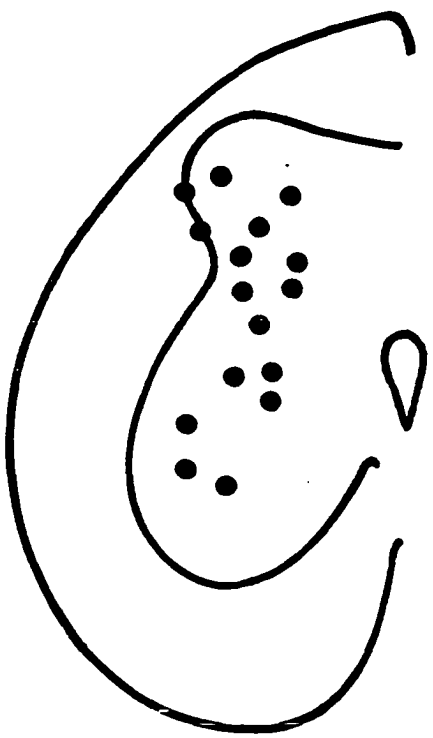

Figure 1. Schematic reconstruction of the location of neurones, in p-CPA-pretreated cats from which extracellular recordings were made

taneous firing.

Typical excitatory responses to SP of 3 units recorded in the p-CPA-pretreated intact cat spinal cord, and activated by both nociceptors and sensitive mechanoreceptors (Fig. 2A), or by sensitive mechanoreceptors (Fig. 2B) or by proprioceptors (Fig. 2C) are illustrated in Fig. 2. As seen in Fig. 2A, a continuous application of SP (100 nA) for about 18 sec produced an excitation within 10 sec and relatively brief afterdischarge. Second application of SP (100 nA) produced an excitation with similar characteristics but with somewhat longer latency. Histological examination revealed the neuron to be located in lamina V (Fig. 2A insert). Another example of the excitatory response to SP of a class 1 unit is illustrated in Fig. 2B. Here a continuous application of SP (60 nA) for 30 sec caused an excitation within 28 sec and an afterdischarge lasting about 2 min. Histological examination revealed the neuron to be located in lamina IV (Fig. 2B insert). The unit illustrated in Fig. 2C had high background activity and was activated by a gentle upward movement of the tail. A continuous application of SP (50 nA) for about 2.5 min caused excitation within 10-15 sec which outlasted application for more than 3 min. On histological examination of the recording site, we found that the unit was located deep in the ventral horn, as illustrated in insert of Fig. 2C.

In a control group of cats (n=3), the mean lumbosacral spinal 5-HT content was 0.711 $\pm$ 0.108 $\mu g/g$ (mean $\pm$ S.E.M.) fresh tissue (Table 2), which value agrees reasonably well with estimates of the 5-HT content of rat (Vogt, 1974) and cat spinal cord (Oliveras et al., 1977). Three days after pretreatment with a single dose of p-CPA (350 mg/kg i.p.), there was

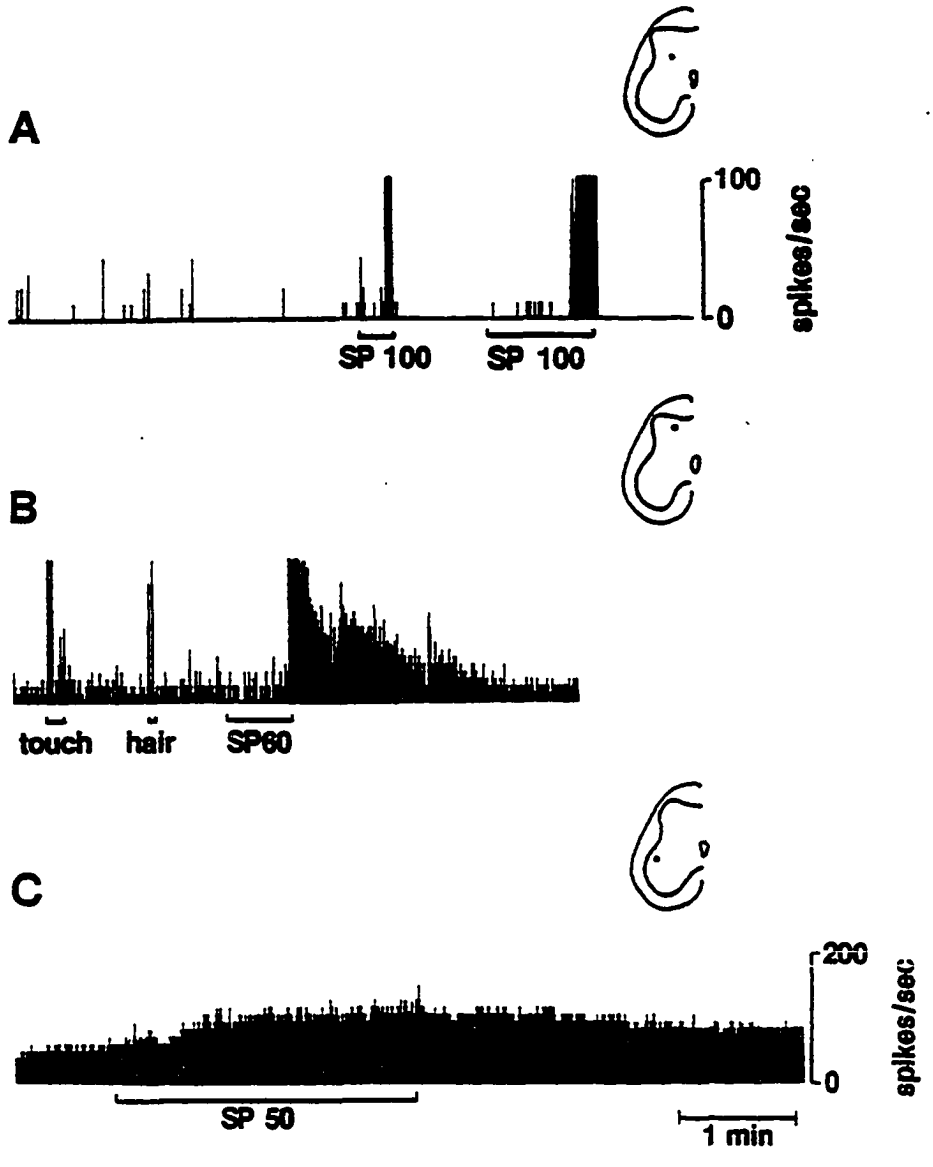

Figure 2. Typical excitatory responses to SP of 3 units recorded in the intact spinal cord of p-CPA-pretreated cat, and activated by both sensitive mechanoreceptors and nociceptors (A), or by sensitive mechanoreceptors (B), or by an input in proprioceptors (C). Rate-meter records of unitary activity in the p-CPA-treated cat spinal cord. Location of neurons from which extracellular recordings were made shown in the insert of Figs. 2A, B, C

a marked decrease in the 5-HT content of the lumbosacral spinal cord. Thus, the 5-HT content of the dorsal half of the spinal cord was lowered to 15.4% (0.111 ± 0.019 μg/g) of the control level, and 18.7% (0.132 ± 0.014 μg/g) in the ventral half of the spinal cord. The levels of SP, CCK, VIP and NT were not consistently changed in p-CPA-pretreated animals.

Table 2. Concentration of 5-HT (μg/g fresh tissue, mean ± S.E.M.) in lumbosacral spinal cord

	Number of animals	Dorsal horn	Ventral horn
Intact cats	3	0.715 ± 0.144	0.707 ± 0.072
p-CPA (350 mg/kg i.p.) treated cats	5	0.111 ± 0.019*	0.132 ± 0.014**

*$P < 0.01$.

**$P < 0.001$.

In conclusion, we have demonstrated in this report: (1) the level of 5-HT was decreased to about 15% of the control value, in the lumbosacral spinal cord, after p-CPA pretreatment, while the levels of SP, CCK, VIP and NT were unchanged; (2) iontophoretic application of synthetic SP caused strong excitation of 31% of class 1 units and 80% of classes 2 and 3 units, and confirmed the results obtained by Randić and Miletić (1977); and (3) SP excited all units receiving cutaneous input and all but one proprioceptive units in the spinal cord of p-CPA-pretreated cats.

SECTION IV. NOREPINEPHRINE REDUCES EXCITABILITY OF SINGLE CUTANEOUS PRIMARY AFFERENT C-FIBERS IN THE CAT SPINAL CORD[1]

A high density of catecholamine-containing nerve terminals of the lower brain stem origin is present in the superficial laminae of the mammalian spinal dorsal horn (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Nygren and Olson, 1977; Satoh et al., 1977; Karoum et al., 1980), the area where unmyelinated primary afferent fibers are shown to terminate (Light and Perl, 1979; Ralston and Ralston, 1979). Norepinephrine (NE) has a potent inhibitory action when applied iontophoretically near spinal dorsal horn interneurons (Engberg and Ryall, 1966; Satoh et al., 1977; Belcher et al., 1978; Headley et al., 1978). This spinal action of NE has been frequently associated with descending inhibitory effects on spinal pain-transmission neurons produced by stimulation in the brain stem areas known to contain catecholamine-containing perikarya (Segal and Sandberg, 1977; Akaike et al., 1978; Belcher et al., 1978; Basbaum and Fields, 1979; Satoh et al., 1980). In addition, a more general tonic brain stem inhibition of C-fiber inputs to dorsal horn interneurons has been reported (Handwerker et al., 1975). The precise neuronal mechanism of these descending inhibitions of spinal dorsal horn interneurons is at present unknown. Although iontophoretically applied NE is shown to inhibit dorsal horn interneurons, it is not clear whether the effect is mediated presynaptically or postsynaptically or both. To

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test more directly for a possible presynaptic action of NE, we have used a modification (Schmidt et al., 1967; Carstens et al., 1979, 1981; Randić, 1981) of Wall's method (Wall, 1958) to measure excitability changes of single sural C-fibers during local administration (iontophoresis and/or pressure microinjection) of NE at the intraspinal sites of lowest threshold for their antidromic activation. Changes in threshold for antidromic activation of primary afferent fibers are thought to reflect changes in the terminal membrane potential, which in turn influences the amount of transmitter released and synaptic transmission (Schmidt, 1971; Levy, 1980). The preliminary results of our findings have been communicated (Semba et al., 1980; Jeftinija et al., 1981c).

Thirty-seven adult cats initially anesthetized with halothane were used. The spinal cord was transected at the first cervical level. Thereafter, the anesthesia was discontinued and the animal was immobilized with succinylcholine chloride (30 mg/kg/hr, i.v.) and artificially ventilated. Mean arterial blood pressure, end-tidal CO_2 and rectal temperature were maintained within physiological limits. The spinal cord was exposed by laminectomy from segments L4 to S1. Bilateral pneumothorax was routinely made. A small filament cut peripherally and dissected free from the left sural nerve was placed on a monopolar silver wire recording electrode. Single C-fibers in the filament were identified by recording unitary action potentials evoked antidromically by electrical stimulation of an L7 or S1 dorsal rootlet with a platinum ball electrode. The sural afferents were classified as C-fibers on the basis of calculated conduction velocity. For intraspinal stimulation of the sural afferents, a glass-coated platinum wire electrode (20 μm diameter, exposed tip < 15 μm

in length) was introduced into the cord just rostral and medial to the dorsal rootlet and a few single sural fibers were re-identified by electrical stimuli (0.2 msec constant current cathodal square pulses) delivered every 3 sec. Threshold measurements were made once every min. The intraspinal sites of lowest threshold for antidromic activation of the single sural fibers were determined systematically. Antidromic threshold was determined by reducing the stimulus current strength in decrements of about 0.5% per stimulus cycle until the fiber did not respond to two successive stimuli. This current intensity was then defined as threshold. Threshold currents for C-fibers ranged from 4 to 10 μA. After the intraspinal sites of lowest threshold for antidromic activation of the sural afferents were found, the platinum microelectrode was removed, and the fibers were re-identified by antidromic stimulation with a compound microelectrode positioned near the same sites. The compound microelectrode consisted of a glass-coated platinum stimulating electrode and an angled multi-barrel micropipette for iontophoresis and/or pressure microinjection of various drugs. Stimulating and drug-applying electrodes were aligned under microscope so that their tips were less than 10 μm apart and were glued together by fast setting epoxy resin. One barrel of the multibarrelled pipette regularly contained either sodium tartrate (0.2 M, pH 4.2-4.7, Malinckrodt) or sodium chloride (0.5-3.0 M, pH 4.2) and served as a control for possible current effects. Current neutralization procedure was also used. Another barrel contained Fast Green solution for marking of intraspinal stimulation sites of C-fibers and application sites of NE. Drug solutions for iontophoresis and/or pressure microinjection were: NE bitartrate

or HCl (0.2 M, pH 3.8-4.7, Regis), phentolamine mesylate (0.05-0.2 M, pH 4.2-4.6, Ciba), yohimbine HCl (0.013 M, pH 3.8, Sigma) and naloxone HCl (0.05 M, pH 4.5, Endo). Naloxone (0.1-0.3 mg/kg) and yohimbine (0.2-1.0 mg/kg) were also administered intravenously. Drug application was begun when threshold values for antidromic activation of C-fibers did not vary by more than $\pm$5% over a period of 5 min, and the mean of these values was taken as a 100% baseline. All threshold values before and during drug application were normalized to this level. Only values during the first 3 min of drug application were used. The data were statistically analyzed by using the one-tail t-test and trend analysis.

Results were obtained from 65 sural afferent C-fibers with conduction velocities ranging from 0.7 to 1.5 m/sec. The anatomical distribution of antidromic stimulation sites of C-fibers and local application sites of NE within the dorsal horn is shown in Fig. 1. The lowest thresholds for antidromic activation of sural C-afferents were found predominantly in laminae I-III, as determined histologically by extracellular dye deposition at the stimulation sites. Iontophoretic application of NE (15-100 nA, for periods of 3 min) resulted in increases in threshold for antidromic activation of most (44/65) C-fibers tested. This effect was dose-dependent and statistically significant, as shown in Table 1. In addition, a few fibers showed reproducible threshold decreases (n=3), some dose-related biphasic effects (n=5), and some were unaffected (n=13) by iontophoretically applied NE. In the fibers which reacted to NE with biphasic responses, there was a tendency for smaller currents (25-50 nA) to reduce threshold, while larger NE currents (100 nA) elevated threshold.

Table 1. Effects of NE on threshold for antidromic activation of sural afferent C-fibers

Drug	Type of Response	Total Number of Fibers	Ejecting Currents: 25 nA/3 min n	25 nA/3 min Mean ± S.E.M.	50 nA/3 min n	50 nA/3 min Mean ± S.E.M.	100 nA/3 min n	100 nA/3 min Mean ± S.E.M.
NE	Increase[a,b]	44	15	111.65 ± 1.52	37	117.19 ± 1.69**	11	121.69 ± 6.29*
Sodium		38			20	102.86 ± 0.74	20	104.04 ± 1.33

[a]At each ejecting current strength NE effects were statistically analyzed against sodium effects by using Student's t-test. (*$P < 0.01$; **$P < 0.001$).

[b]In the fibers responding to NE with threshold increase dose-dependency was statistically significant by a trend analysis ($F = 4.30$, $df = 1, 60$, $P < 0.05$).

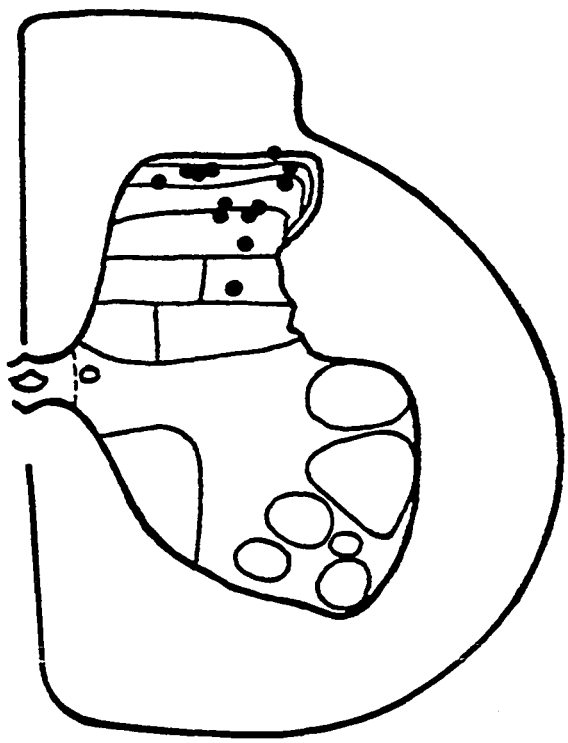
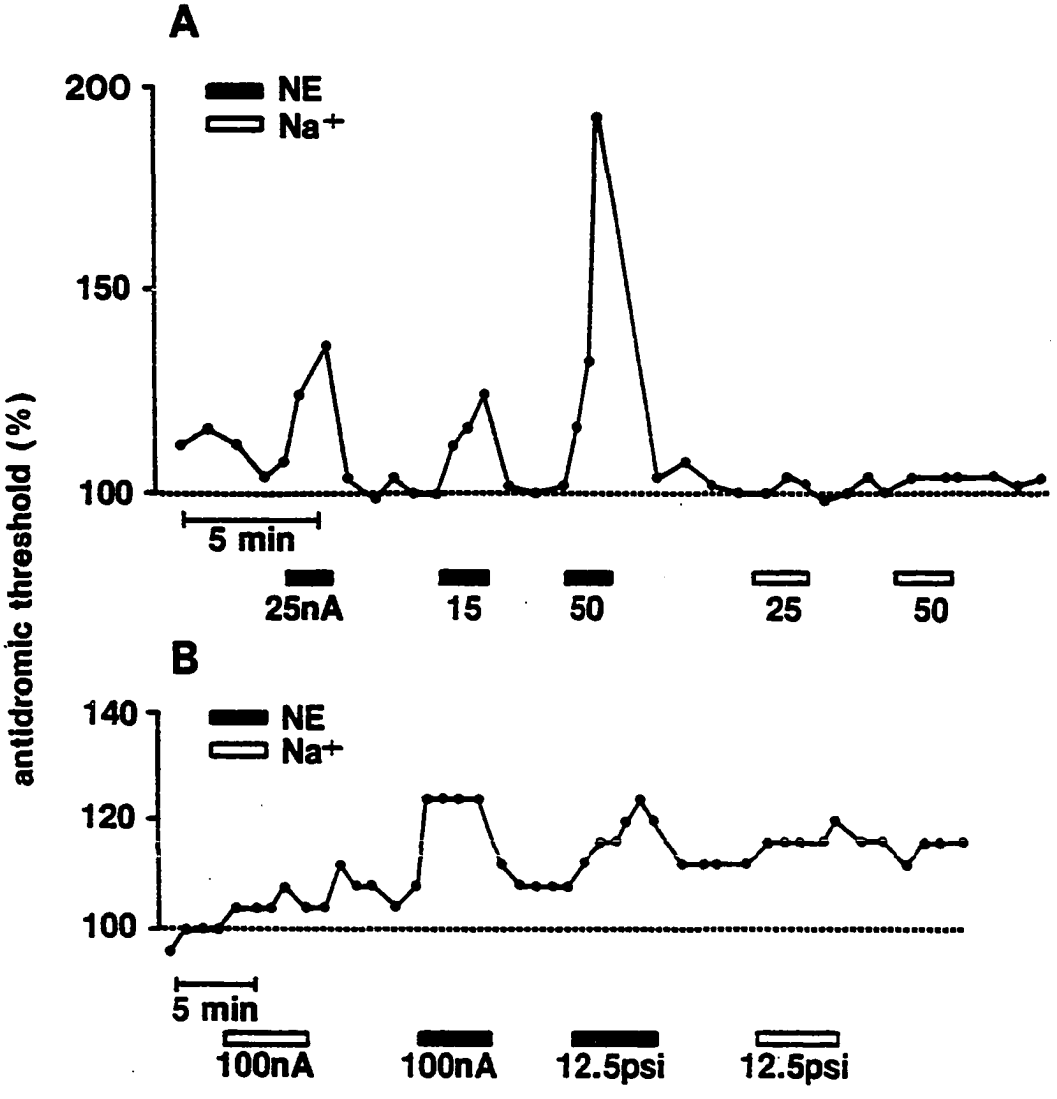


Figure 1. A transverse hemisection of L_7 spinal segment illustrating the location of stimulating sites in the dorsal horn where local application of norepinephrine elevated the threshold for antidromic activation of single sural afferent C-fibers

Average threshold increases in 44 C-fibers during NE application (50 nA/3 min) ranged from 106-148% of control. Threshold increases during NE applications with 25, 50, and 100 nA were dose-related (trend analysis, $F = 4.30$; $df = 1.60$; $P < 0.05$). In general, threshold increases began within 30 sec following the onset of NE application, reached a maximum within 2 min and completely recovered within 1-2 min after termination of NE current. An example of the dose-related threshold increases produced by NE in a single C-fiber is illustrated in Fig. 2A. Here iontophoretic administration of NE with 50 nA current for 108 sec produced an increase in the peak threshold value to about 190% of the control level. Smaller but dose-related threshold increases were produced by NE at lower current strengths (i.e. to 137% with 25 nA and to 126% with 15 nA). Since the actual passage of outward current may affect the level of membrane polarization in intraspinal C-fibers, it was important to distinguish between the effects of NE itself, and the associated current flow. As it can be seen from Fig. 2A, the outward currents of 25 and 50 nA applied through the control barrel (sodium ions) apparently did not influence the threshold. Elevation in the threshold for antidromic activation was also observed in another sural C-fiber when NE was applied by pressure microinjection (Fig. 2B).

It has not been possible to produce any convincing evidence of a blockade of the action of NE on C-fibers, either by iontophoretically applied phentolamine (n=3) or both locally and intravenously administered yohimbine (n=6). However, these agents frequently per se produce changes in the threshold for antidromic activation of C-fibers. In addition, in no instance was the action of NE antagonized to any

Figure 2. Typical examples of increases in threshold for antidromic activation of two different C-fibers produced by intraspinally applied norepinephrine. Control ejection of sodium ions was without comparable effect. A: Normalized antidromic threshold measurements in a single sural C-fiber prior to and during iontophoretic application of NE (15, 25 and 50 nA) and sodium ions (25, 50 nA). Durations of application are indicated by black (NE) and white (sodium) bars. Note the dose-dependent effects with NE, and lack of effect with sodium ions. B: Normalized antidromic threshold values for another C-fiber during iontophoretic (100 nA) and pressure microinjections (12.5 psi) of NE or sodium ions. Note the comparable effects produced by the two means of administration, and lack of effect with sodium tartrate


A
200
150
100
NE
Na+
5 min
25nA
15
50
25
50
antidromic threshold (%)
B
140
120
100
NE
Na+
5 min
100nA
100nA
12.5psi
12.5psi

significant degree by naloxone, either ejected locally or administered intravenously (n=10).

Threshold reductions down to 93% of control were observed in 6 out of 14 C-fibers tested by conditioning stimulation of the posterior tibial nerve at a strength supramaximal for C-fibers, (25 V, 1 msec, conditioning-test interval 250 msec).

Our results indicate that local administration of NE into the superficial layers of the spinal dorsal horn reduces the electrical excitability of about 68% of sural afferent C-fibers tested. In addition, about 5% of tested fibers showed reproducible increase in excitability, 8% dose-related biphasic effects, and the rest (19%) were unaffected. The extent to which these actions of NE were confined to the nerve terminals or involved the preterminal axons as well cannot be determined from our experiments.

In discussing these effects in terms of a possible presynaptic action of NE, it is important to consider other factors which might have contributed to such threshold changes. Although the method of local application of chemicals, either by iontophoresis or pressure microinjection, offers some advantages as compared with the systemic route, iontophoretic administration of a chemical may produce changes in extracellular resistance due to ion fluxes and alterations in extracellular space; the latter in turn may change the threshold for antidromic activation of a fiber as the recent measurements with ion-sensitive electrodes have demonstrated (Krnjević et al., 1980; Segal and Gutnick, 1980). However, in our experiments numerous current controls (ejection of Na^+, Cl^- or tartrate ions) had little or no effect on

C-fiber thresholds, whereas NE application generally produced threshold changes which were graded with the strength of the ejection current. NE effects were also present when current neutralization procedure was employed. This technique rules out the possibility that the polarization level of fibers is changed after their conductance has been altered by the drug. In addition, NE effects were reproduced when this drug, but not sodium chloride or tartrate, was applied by pressure microinjection.

In relation to recent reports suggesting that alpha-adrenergic receptors play a major role in mediating the effects of norepinephrine in the spinal cord (Kuraishi et al., 1979a; Reddy and Yaksh, 1980; Reddy et al., 1980), we have examined effects of yohimbine and phentolamine on the NE-produced reduction of the electrical excitability of sural C-fibers. It has not been possible to produce any convincing evidence of a blockade of the threshold increase in C-fibers, either by iontophoretically applied phentolamine and yohimbine or by systemically applied yohimbine in concentrations which had no effect on the excitability of sural afferents and systemic blood pressure. However, when phentolamine was ejected with larger iontophoretic currents (i.e. > 10-20 nA/5-10 min) the threshold for antidromic activation of C-fibers gradually increased and slowly recovered. Thus, it may not be possible to achieve by the iontophoretic technique an adequate concentration of the blocking agent at the receptors without causing this undesirable action on the excitability of C-fibers at the same time.

Because of the evidence for the involvement of adrenergic mechanisms in modulation of spinal processing of nociceptive information (Belcher

et al., 1978; Headley et al., 1978; Kuraishi et al., 1979a,b; Reddy and Yaksh, 1980; Reddy et al., 1980; Tyce and Yaksh, 1981), and the presence of leu-enkephalin (Elde et al., 1976) and opiate receptors in the dorsal horn (Atweh and Kuhar, 1977) and dorsal roots (LaMotte et al., 1976; Fields et al., 1980) it was of interest to test effects of naloxone on the depressant action of NE on the excitability of C-fibers. As our data show, naloxone had no antagonistic effect on the threshold increase produced by NE in C-fibers suggesting that this action of NE is not likely to be mediated either by enkephalin-containing interneurons or by opiate receptors present on primary afferents.

It cannot be determined with our method whether NE acts directly at the intraspinal afferent C-fibers and/or indirectly via inhibition of dorsal horn interneurons possibly mediating presynaptic excitability changes at the C-fibers. In addition, we cannot exclude the possibility that NE effects were produced through non-synaptic mechanisms (Owman and Santini, 1966; Chan-Palay, 1978). However, it is of relevance that the intraspinal antidromic thresholds of some polymodal nociceptors and high-threshold mechanoreceptors with afferent C-fibers were shown to be elevated during electrical stimulation of the brain stem areas containing monoamines (Hentall and Fields, 1979; Carstens and Zimmermann, 1981). These findings coupled with available morphological (LaMotte et al., 1976; Ralston and Ralston, 1979; Ruda et al., 1979), histochemical (Hökfelt et al., 1976), and pharmacological (Carstens et al., 1979, 1981; Sastry, 1979a; Randić, 1981) data seem to suggest existence of presynaptic inhibitory control of C-fibers. Therefore, threshold increases observed with NE may represent a presynaptic inhibition of synaptic transmission

at C-fiber terminals. Dunlap and Fischbach (1979) have recently observed that NE decreases the inward calcium current of the soma membrane in cultured embryonic chick sensory neurons, the result if it were to obtain in presynaptic terminals might provide a novel mechanism for presynaptic modulation of transmitter release.

In conclusion, our data suggest that activation of catecholaminergic descending pathways may exert a modulating influence over the processing of sensory information carried by curaneous C-fibers into the spinal cord.

We would like to thank Drs. D. Egger and H. Geller for their helpful comments on the manuscript.

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SECTION V: NOREPINEPHRINE REDUCES EXCITABILITY OF SINGLE CUTANEOUS PRIMARY AFFERENT C- AND A-FIBERS IN THE CAT SPINAL CORD[1]

A high density of catecholamine-containing nerve terminals of the lower brain stem origin is present in the superficial laminae of the mammalian spinal dorsal horn (Dahlström and Fuxe, 1965; Nygren and Olson, 1977), the area where unmyelinated primary afferent fibers are shown to terminate (Light and Perl, 1979; Ralston and Ralston, 1979). Norepinephrine (NE) has a potent inhibitory action when applied iontophoretically near spinal dorsal horn interneurons (Engberg and Ryall, 1966; Belcher et al., 1978). This spinal action of NE has been frequently associated with descending inhibitory effects on spinal pain-transmission neurons produced by stimulation in the brain stem areas known to contain catecholamine-containing perikarya (Segal and Sandberg, 1977; Akaike et al., 1978; Basbaum and Fields, 1979). In addition, a more general tonic brain stem inhibition of C-fiber inputs to dorsal horn interneurons has been reported (Handwerker et al., 1975). Although iontophoretically applied NE is shown to inhibit dorsal horn interneurons, it is not clear whether the effect is mediated presynaptically or postsynaptically or both.

To test more directly for a possible presynaptic action of NE, we have used a modification (Schmidt et al., 1967) of Wall's method (Wall, 1958) to measure excitability changes of single sural C- and A-fibers during

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local administration of drugs at the intraspinal sites of lowest threshold for their antidromic activation. The preliminary results of our findings have been communicated (Semba et al., 1980).

Forty-four adult cats initially anesthetized with halothane and later spinalized were used. The spinal cord was exposed by laminectomy from segments L4 to S1. Small filaments were dissected free from the sural nerve and placed on a monopolar silver wire recording electrode. Single fibers in the filament were identified by recording unitary action potentials evoked antidromically by electrical stimulation of an L7 or S1 dorsal root. The sural afferents were classified as C- and A-fibers on the basis of calculated conduction velocity. For intraspinal stimulation of the sural afferents, a glass-coated platinum wire electrode (20 μm diameter, exposed tip <15 μm in length) was used. Stimuli (0.2 msec constant current cathodal square pulses) were delivered every 3 sec. Antidromic threshold was determined by reducing the stimulus current strength in decrements of about 0.5% per stimulus cycle until the fiber did not respond to two successive stimuli. This current intensity was then defined as threshold. Threshold currents for C-fibers ranged from 4 to 10 μA, for Aδ-fibers from 2 to 5 μA, and for Aβ-fibers from 1 to 4 μA. After the intraspinal site of lowest threshold for antidromic activation of the sural afferent was found, a compound microelectrode was positioned near the same site. The compound microelectrode consisted of a glass-coated platinum stimulating electrode and an angled multi-barrel micropipette for iontophoresis and/or pressure microinjection of various drugs. One barrel of the multibarrelled pipette regularly contained either sodium tartrate (0.2 M, pH 4.2-4.7, Malinckrodt) or

sodium chloride (0.5-3.0 M. pH 4.2) and served as a control for possible current effects. Current neutralization procedure was also used. Drug solutions were: NE bitartrate or HCl (0.2 M, pH 3.8-4.7, Regis), clonidine HCl (0.2 M, pH 4.2, Boehringer), isoproterenol bitartrate

Table 1. The effects of norepinephrine and other sympathomimetic drugs on threshold for antidromic activation of sural afferent C- and A-fibers

Drug	C-fibers			A-fibers		
	n[a]	Increase	No effect	n	Increase	No effect
Norepinephrine	65	44	13	9	8	1
Dopamine	9	3	6			
Clonidine	4	3	1	4	4	0
Isoproterenol	2	2	0	4	3	1
Phenylephrine	5	5	0	6	4	2

[a] n = number of fibers tested.

(0.2 M, pH 4.2, Sigma), phenylephrine HCl (0.2 M, pH 4.2, Sigma), phentolamine mesylate (0.05-0.2 M, pH 4.2-4.6, Ciba), yohimbine HCl (0.013 M, pH 3.8, Sigma), dopamine HCl (0.2 M, pH 4.2, Regis), and naloxone HCl (0.05 M, pH 4.5, Endo). Naloxone (0.1-0.3 mg/kg) and yohimbine (0.2-1.0 mg/kg) were also administered intravenously. The NE data were statistically analyzed by using the t-test.

Results were obtained from 65 C-fibers (conduction velocity <1.5 m/sec), 5 Aδ-fibers (5-20 m/sec), and 4 Aβ-fibers (>40 m/sec). They are presented in Table 1. Iontophoretic application of NE (15-100 nA,

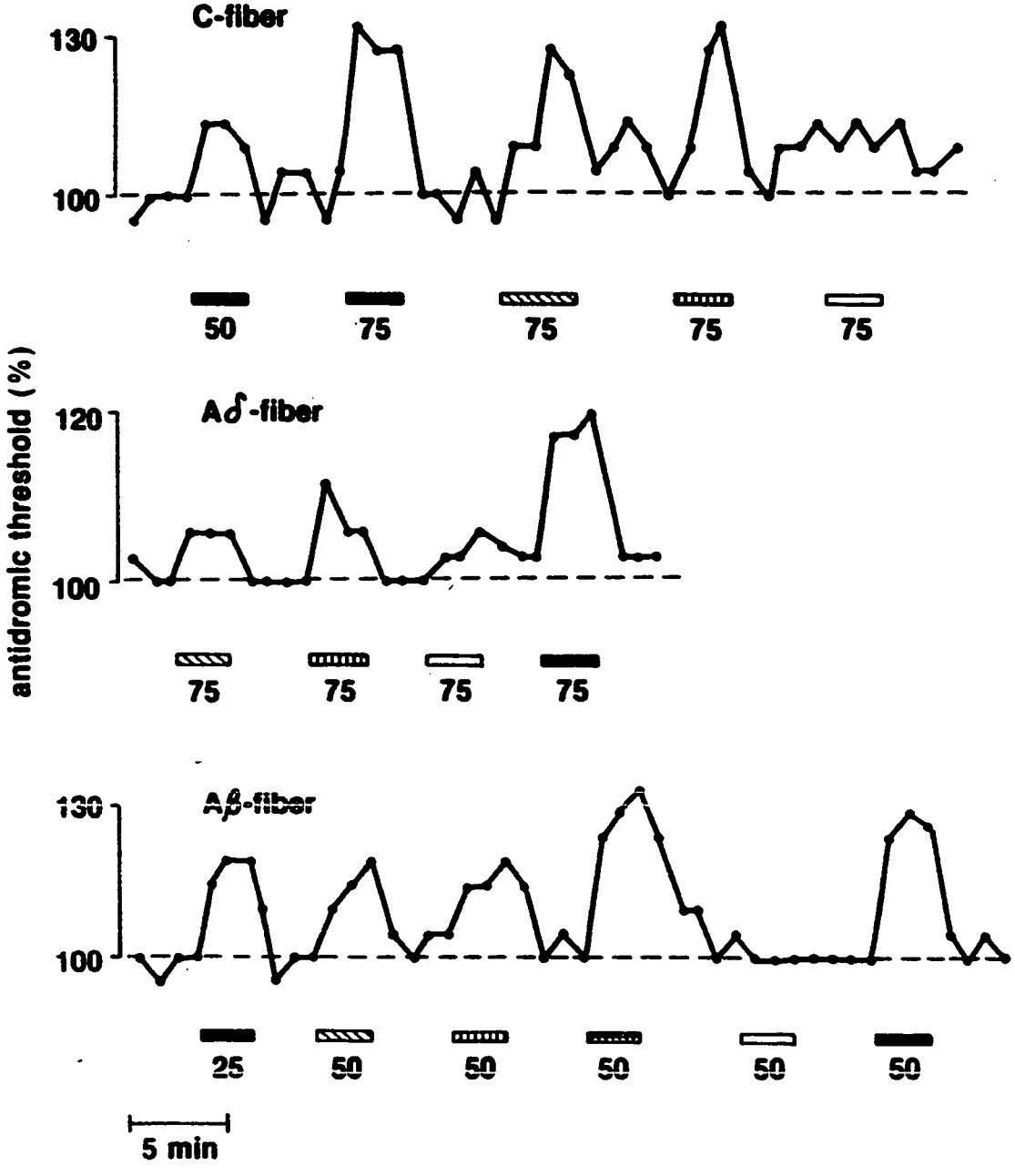
for periods of 3 min) resulted in increases in threshold for antidromic activation of most (44/65) C-fibers tested. The mean threshold increase for C-fibers during NE-application (to 117.2 $\pm$ 1.7 S.E.M. of control with 50 nA/3 min) was statistically significant ($P < 0.001$). In general, threshold increases began within 30 sec following the onset of NE application, reached a maximum within 2 min, and completely recovered within 1-2 min after termination of NE current. An example of the dose-related threshold increases produced by NE in a single C-fiber is illustrated in Fig. 1. In addition, a few C-fibers showed reproducible threshold decreases (n=3), some dose-related biphasic effects (n=5), and some were unaffected (n=13) by iontophoretically applied NE.

As shown in Table 1, clonidine, dopamine, isoproterenol, and phenylephrine also produced increases in threshold for antidromic activation in a proportion of C- and A-fibers tested.

It has not been possible to produce any convincing evidence of a blockade of the action of NE on C-fibers, either by iontophoretically applied phentolamine (n=3) or both locally and i.v. administered yohimbine (n=6). In addition, in no instance was the action of NE antagonized to any significant degree by naloxone, either ejected locally or administered i.v. (n=10).

Threshold reductions down to 93% of control were observed in 6 out of 14 C-fibers tested by conditioning stimulation of the posterior tibial nerve at a strength supramaximal for C-fibers (25 V, 1 msec, conditioning test interval 250 msec). In addition, we have found in 12 C-fibers tested with conditioning stimuli of the posterior tibial nerve with cathodal pulses at A-fiber strength (2 V, 0.1 msec, conditioning

Figure 1. Typical examples of increases in threshold for antidromic activation of sural C-fiber (top record), Aδ-fiber (middle record), and Aβ-fiber (bottom record) produced by intraspinally applied norepinephrine (▬), clonidine (▭), isoproterenol (▭), and phenylephrine (▭). Top record: normalized antidromic threshold measurements in a single C-fiber prior to and during iontophoretic application of NE (50, 75 nA), isoproterenol (75 nA), phenylephrine (75 nA), and sodium ions (▭, 75 nA). Note the dose-dependent effects with NE, and lack of effect with sodium ions. Middle record: normalized antidromic threshold measurements in a single Aδ-fiber prior to and during iontophoretic application of norepinephrine (75 nA), isoproterenol (75 nA), phenylephrine (75 nA), and sodium ions (75 nA). Norepinephrine appeared to be the most potent agent in reducing the excitability of Aδ-fiber. Sodium ions had little effect. Bottom record: threshold measurements in a single Aβ-fiber prior to and during iontophoretic application of norepinephrine (25, 50 nA), clonidine (50 nA), isoproterenol (50 nA), phenylephrine (50 nA), and sodium ions (50 nA). Note that clonidine appeared to be the most potent agent in reducing the excitability of Aβ-fiber


C-fiber
130
100
50
75
75
75
75
antidromic threshold (%)
Aδ-fiber
120
100
75
75
75
75
Aβ-fiber
130
100
25
50
50
50
50
50
5 min

test interval 30-50 msec) that stimulation produced either a decrease in threshold (n=5) or had no effect (n=7).

Our results indicate that local administration of NE into the superficial layers of the spinal dorsal horn reduces the electrical excitability of about 68% of sural afferent C-fibers tested. In addition, about 5% of tested fibers showed reproducible increase in excitability, 8% dose-related biphasic effects, and the rest (19%) were unaffected.

In relation to recent reports suggesting that α-adrenergic receptors play a major role in mediating the effects of NE in the spinal cord (Kuraishi et al., 1979a; Reddy et al., 1980), we have examined effects of α- and β-adrenergic receptor agonists and α-receptor antagonists on the excitability of sural afferents and the NE-produced threshold increase in C- and A-fibers, respectively. We have found that both α- and β-receptor agonists cause a reduction of the excitability of C- and A-fibers, clonidine being the most potent in this respect and isoproterenol the least potent. It has not been possible to produce any convincing evidence of a blockage of the NE-induced threshold increase in C-fibers, either by iotophoretically applied phentolamine and yohimbine or by i.v. applied yohimbine in concentrations which had no effect on the excitability of sural afferents and systemic blood pressure.

Because of the evidence for the involvement of adrenergic mechanisms in modulation of spinal processing of nociceptive information (Belcher et al., 1978; Headley et al., 1978; Kuraishi et al., 1979a; Reddy et al., 1980) and the presence of enkephalin and opiate receptors in the dorsal

horn (Atweh and Kuhar, 1977) and the dorsal roots (LaMotte et al., 1976), it was of interest to test effects of naloxone on the depressant action of NE on the excitability of C-fibers. As our data show, naloxone had no antagonistic effect on the threshold increase produced by NE in C-fibers suggesting that this action of NE is not likely to be mediated either by enkephalin-containing interneurons or by opiate receptors present on primary afferents.

It cannot be determined with our method whether NE acts directly at the intraspinal afferent C-fibers and/or indirectly via inhibition of dorsal horn interneurons possibly mediating presynaptic excitability changes at the C-fibers. Dunlap and Fischbach (1979) have recently observed that NE decreases the inward calcium current of the soma membrane in cultured sensory neurons, the result if it were to obtain in presynaptic terminals might provide a novel mechanism for presynaptic modulation of transmitter release. Recent pharmacological data seem to support the concept of presynaptic control of C-fibers (Carstens et al., 1979; 1981; Jeftinija and Randić, 1981; Randić, 1981).

In conclusion, our data suggest that activation of catecholaminergic descending pathways may exert a modulating influence over the processing of sensory information carried by cutaneous C- and A-fibers into the spinal cord.

This work was supported by the National Science Foundation (BNS 23871) and the United States Department of Agriculture.

SECTION VI. SOMATOSTATIN AND GLUTAMIC ACID INCREASE EXCITABILITY OF SINGLE CUTANEOUS PRIMARY AFFERENT C- AND A-FIBERS IN THE CAT SPINAL CORD

Immunohistochemical studies demonstrated the presence of somatostatin in small neuronal cell bodies of the spinal ganglia and in the neuronal fibers within the spinal dorsa horn (Hökfelt et al., 1975a). Release of somatostatin from central terminals of primary sensory neurons has been observed in vitro (Mudge et al., 1977) and in vivo following activation of small diameter primary afferents (Jessell et al., 1979a). Although intracellular recordings revealed that somatostatin hyperpolarizes dorsal horn neurons indicative of a postsynaptic action (Murase and Randić, 1981; Murase et al., 1982) a presynaptic site of action of somatostatin was observed on the cultured spinal cord neurons (Macdonald and Nowak, 1981a) and dorsal root ganglion cells (Dunlap and Fischbach, 1978). In addition, the response to glutamate was depressed by somatostatin in both dorsal and ventral roots of the frog spinal cord (Padjen, 1977) but potentiated in the sensorimotor cortex of awake rabbits (Ioffe et al., 1978) and cortical neurons kept in the cell culture (Dichter and Delfs, 1981). The latter results suggested that somatostatin may modulate excitatory glutamate responses.

In the spinal cord, L-glutamate concentration was found to be the highest in the dorsal gray area where primary afferent fibers terminate (Graham et al., 1967; Johnson and Aprison, 1970). Glutamate has an excitatory action when applied iontophoretically near spinal cord neurons (Curtis et al., 1960; Zieglgänsberger and Puil, 1973a; Engberg et al., 1979). In addition, a depolarizing effect of L-glutamate on the large primary afferent fibers has been shown (Eccles et al., 1963b; Schmidt, 1963; Curtis and

Ryall, 1966). The results obtained by De Groat et al. (1972) that L-glutamate does not have an effect on dorsal root ganglion cells, and further confirmations of a depolarizing action of L-glutamate on the central terminals of primary afferent neurons in both mammalian and amphibian species (Barker et al., 1975; Curtis et al., 1977; Evans, 1978) lead to the conclusion that receptors for L-glutamate are confined exclusively to the central terminals of primary afferent neurons. However, on the basis of his data obtained in the frog, Evans (1980) proposed an alternative explanation for the depolarizing effect of L-glutamate on primary afferent fibers, namely that they do not possess excitatory amino acids receptors but are depolarized indirectly through the release of K ions from excited dorsal horn neurons.

To test more directly for a possible presynaptic action of somatostatin and L-glutamate we have used a modification (Schmidt et al., 1967) of Wall's method (Wall, 1958) to measure excitability changes of single sural A- and C-fibers during iontophoretic administration of somatostatin and L-glutamate at the intraspinal sites of the lowest threshold for their antidromic activation. The preliminary results of our findings have been communicated (Jeftinija and Randić, 1981).

Thirteen adult cats (2.0-4.0 kg) initially anesthetized with halothane were used. The spinal cord was transected at the first cervical level. The surgical, stimulation and recording procedures were identical to those described in Sections IV and V. Drugs were administered in the immediate vicinity of intraspinal primary afferent fibers. For iontophoresis somatostatin (3.1 mM, pH 5.5, Beckman) was dissolved in 20 mM acetic acid and ejected as cation. L-glutamate (1.0 M, pH 7.2, Calbiochem) was dissolved

in 165 mM NaCl and ejected as anion. One barrel of the multibarrelled pipette regularly contained either sodium chloride (165 mM, pH 7.0) or sodium acetate (20 mM, pH 5.5) and served as a control for possible current effects. Another barrel contained Fast Green dye in 3 M sodium chloride for marking of intraspinal stimulation sites of primary afferent fibers and drug application sites. Drug application was begun when threshold values for antidromic activation of sural afferents did not vary by more than $\pm$5% over a period of 5 min, and the mean of these values was taken as a 100% baseline. All threshold values before and during drug application were normalized to this level. Only values during the first 3 min of L-glutamate and 5 min of somatostatin application were used.

The results of the present experiments are shown in Table 1. The effect of somatostatin was tested on 25 C-fibers (conduction velocity < 1.5 m/sec), 11 Aδ-fibers (conduction velocity 5-20 m/sec) and 4 Aβ-fibers (conduction velocity > 40 m/sec). Iontophoretic application of somatostatin

Table 1. The effects of somatostatin (25 to 125 nA/5 min) and L-glutamate (15 to 125 nA/3 min) on threshold for antidromic activation of sural afferent C- and A-fibers

Drug	Type of fiber	Total number of fibers	Threshold		
			Decrease	Increase	No effect
Somatostatin	C	25	12	4	9
	Aδ	11	6	1	4
	Aβ	4	0	1	3
L-glutamate	C	27	15	0	12
	Aδ	13	8	0	5
	Aβ	5	5	0	0

(25 to 125 nA, for periods up to 5 min) resulted in a decrease in threshold for antidromic activation in 12 out of 25 C-fibers and 6 out of 11 Aδ-fibers tested. This effect was dose-dependent and reversible. In addition, 4 C-fibers, 1 Aδ- and 1Aβ-fiber showed a reproducible threshold increase. The rest of C- and A-fibers were unaffected by somatostatin. Mean threshold reductions during the first 5 min of iontophoretic application of somatostatin varied between 67% and 95%. An example of the dose-related threshold decrease produced by somatostatin in a single C-fiber is illustrated in the upper record of Fig. 1. Here, iontophoretic administration of SS with 100 nA current for 200 sec produced a maximum decrease in the threshold value to about 86% of the control level. A smaller but dose-related threshold decrease was produced by somatostatin applied at a lower current strength. Since the actual passage of outward current may affect the level of membrane polarization in intraspinal fibers, it was important to distinguish between the effects of somatostatin itself and the associated current flow. As can be seen from Fig. 1, the outward current of 100 nA applied through the control barrel (sodium ions) produced a slight increase rather than a decrease in the threshold. The lower record in Fig. 1 illustrates threshold reductions in an Aδ-fiber during somatostatin application (75 nA for 4 min) and lack of similar effect with sodium ions.

The effect of L-glutamate was tested on 27 C-fibers, 13 Aδ- and 5 Aβ-fibers (Table 1). Iontophoretic application of L-glutamate (15 to 125 nA, for periods up to 3 min) resulted in a decrease in the threshold for antidromic activation in 15 out of 27 C-fibers, 8 out of 13 Aδ-fibers and all Aβ-fibers tested. This effect was dose-dependent and reversible. Twelve C-fibers and 5 Aδ-fibers were unaffected by iontophoretically applied L-

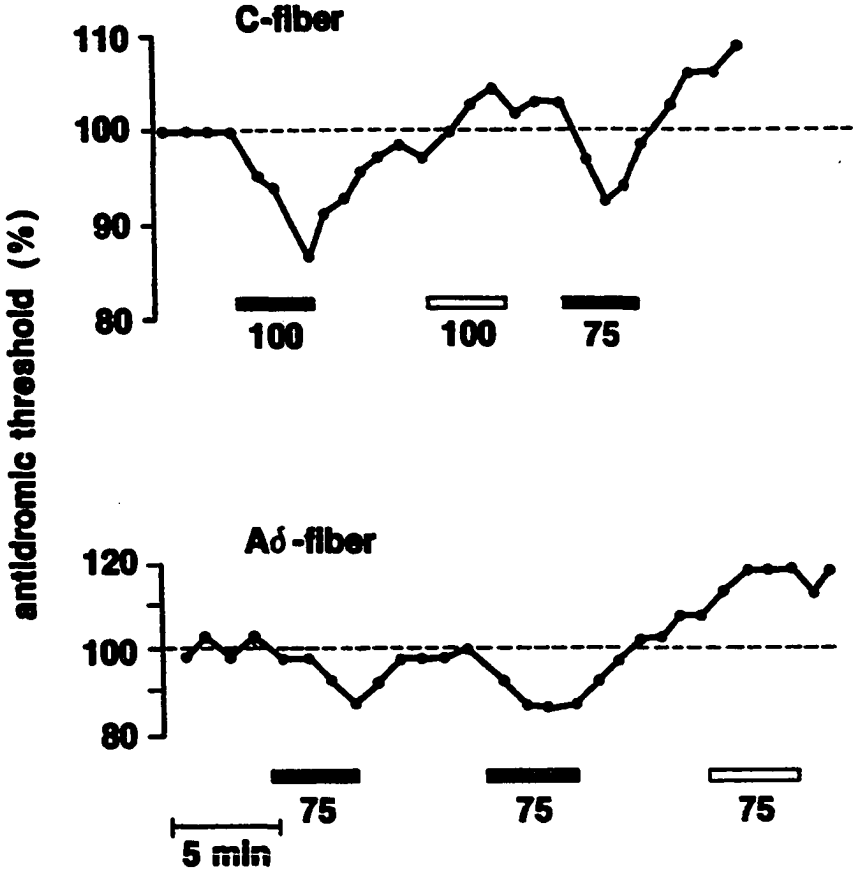


Figure 1. Typical examples of decreases in threshold for antidromic activation of sural C-fiber (upper record) and Aδ-fiber (lower record) produced by somatostatin (▬▬). Upper record: normalized antidromic threshold measurements in a single C-fiber prior to and during intraspinal iontophoretic application of somatostatin (75, 100 nA) and sodium ions (▭, 100 nA). Note the threshold reductions during somatostatin application and lack of effect with sodium ions. Lower record: threshold measurements in a single Aδ-fiber prior to and during iontophoretic application of somatostatin (75 nA) and sodium ions (75 nA)

glutamate. The mean threshold reductions during the first 3 min of iontophoretic L-glutamate application varied between 67% and 93%. An example of the dose-related threshold decreases produced by L-glutamate in a single C-fiber (upper record) and a single Aδ-fiber (lower record) is illustrated in Fig. 2. Here iontophoretic administration of L-glutamate with 50 nA current for 165 sec produced a maximum decrease in the threshold value to about 92% of the control value for C-fiber and to a similar degree for Aδ-fiber. Bigger, but dose-related threshold decreases were produced by L-glutamate at higher current strengths. As can be seen in Fig. 2, the corresponding currents applied through the control barrel (acetate ions) did not alter the threshold.

In several fibers tested, an absence of parallelism in the effects of somatostatin and L-glutamate on the threshold for antidromic activation was observed. An example for this is shown in Fig. 3. The upper record illustrates a normalized antidromic threshold measurements in a single C-fiber in which iontophoretic application of somatostatin (100 nA) for 200 sec had no effect, while L-glutamate (15, 30 and 50 nA) produced a dose-related threshold decrease. The lower record illustrates threshold measurements of a single Aδ-fiber in which iontophoretically applied somatostatin (100 and 50 nA) for a period of 180 sec produced dose-related threshold decrease, while L-glutamate (50 and 100 nA) had no effect.

It has not been possible to produce any convincing evidence of a blockade of the action of L-glutamate on the primary afferent fibers (n=5) by iontophoretic application of bicuculline with currents of 30 to 60 nA applied for 10 to 30 min (bicuculline methiodide 5mM, pH 3.0-3.5, Pierce). In addition, it has not been possible to produce any convincing evidence of

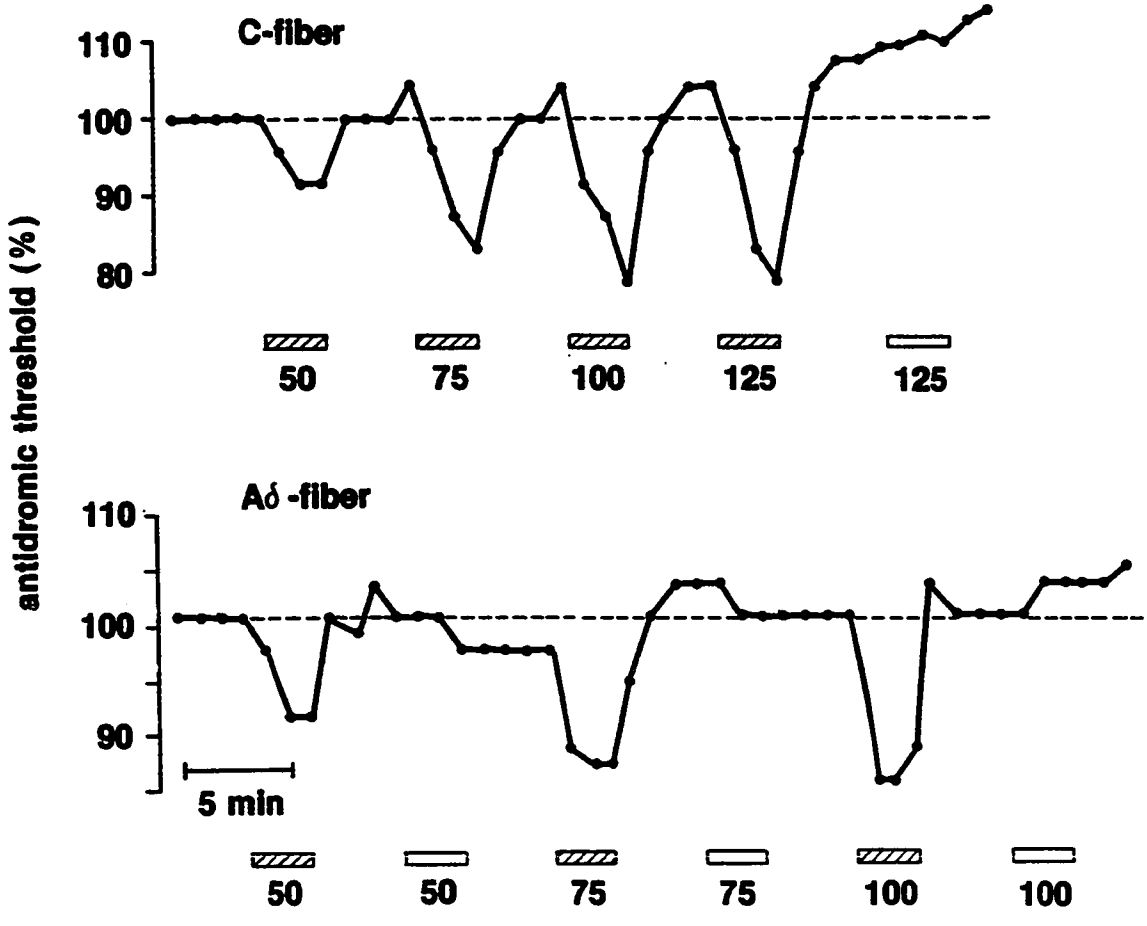


Figure 2. Typical examples of decreases in threshold for antidromic activation of sural C-fiber (upper record) and Aδ-fiber (lower record) induced by L-glutamate (▨). Upper record: normalized antidromic threshold measurements in a single C-fiber prior to and during intraspinal iontophoretic application of L-glutamate (50, 75, 100, 125 nA) and acetate ions (▭ 125 nA). Note the dose-dependent effects with L-glutamate and lack of effect with acetate. Lower record: threshold measurements in a single Aδ-fiber prior to and during iontophoretic application of L-glutamate (50, 75, 100 nA) and acetate ions (50, 75, 100 nA)

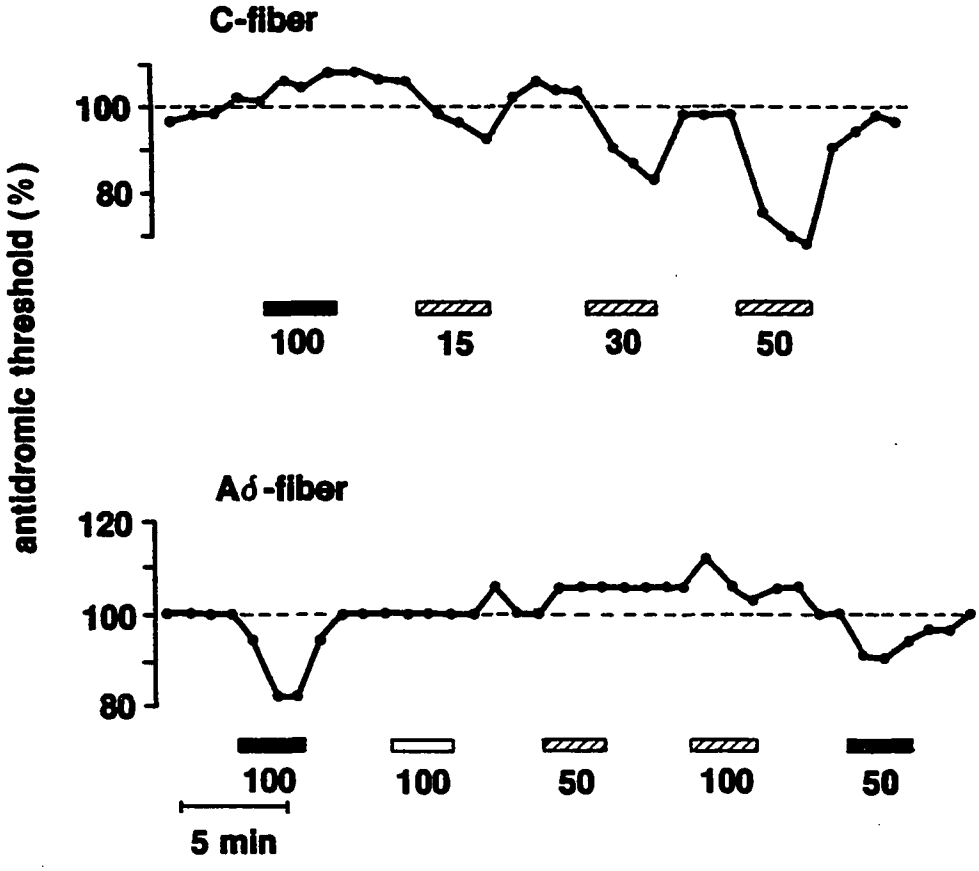


Figure 3. Absence of parallelism of somatostatin (▬) and L-glutamate (▨) effects in the same sural C- and Aδ-fibers. Upper record: normalized antidromic threshold measurements in a single C-fiber prior to and during iontophoretic application of somatostatin (100 nA) and L-glutamate (15, 30, 50 nA). Note the dose-dependent decrease in threshold produced by L-glutamate and lack of effect with somatostatin. Lower record: threshold measurements in a single Aδ-fiber prior to and during iontophoretic application of somatostatin (50, 100 nA), L-glutamate (50, 100 nA) and sodium ions (▭, 100 nA). Note the dose-dependent decrease in threshold produced by somatostatin and lack of effect with L-glutamate

a blockade or potentiation of the action of L-glutamate on primary afferents by iontophoretically applied somatostatin (n=6).

The present results indicate that local administration of somatostatin and L-glutamate increases the electrical excitability of about half of the sural afferenct C- and A-fibers. In addition, about 16% of the C-fibers tested with somatostatin showed a reproducible decrease in excitability. The decrease in excitability was not seen with L-glutamate.

DISCUSSION

For years, the mammalian superficial spinal dorsal horn has drawn attention as being structurally and functionally different from the rest of the spinal gray matter. Some authors see this region of the spinal cord as a receiving station and relay nucleus for information carried by the primary afferent fibers while others suggest a more complex functional role such as modulation of cutaneous sensory information. In order to increase our knowledge about the functional role of the superficial part of the spinal dorsal horn , more information about physiological and pharmacological actions of several compounds known to be present in this area, are needed. Within the past several years, it has been revealed that several neuroactive peptides (e.g. CCK, enkephalin, NT, SS, SP, VIP), biogenic amines (e.g. NE, 5-HT) and amino acids (e.g. GABA, L-glutamate) to which transmitter or modulator functional roles have been attributed, are present in the superficial spinal dorsal horn. The interest of investigators in this region is now focused on the potential role of these substances in the integration and distribution of sensory information arriving into the spinal dorsal horn.

The excitatory actions of SP and NT and depressant actions of SS and enkephalin on the cat dorsal horn neurons in laminae I-III have been, earlier observed in our laboratory (Randić and Miletić, 1977, 1978; Miletić and Randić, 1979). In this thesis, we demonstrate that CCK-8 and VIP have excitatory actions on spinal neurons located in laminae I-VII of the cat spinal cord. The excitation produced by CCK-8 and VIP was observed in all categories of neurons recognized in the spinal preparations of cats

on the basis of their excitability by different kinds of cutaneous afferent input. The finding made in the spinal cord slice preparation that the excitatory action of CCK-8 persisted even when the slices were perfused with Ca^{2+}-free, Mg^{2+}-high solution, indicates that CCK-8 may act directly on the postsynaptic membrane. Intracellular recordings from single neurons in the same in vitro preparation showed that VIP depolarizes dorsal horn neurons and increases their excitability.

A number of reports appeared recently showing that SP, opioids, 5-HT and GABA besides affecting excitability of dorsal horn neurons, also modify excitability of intraspinal cutaneous C- and A-fibers (Sastry, 1979a; Carstens et al., 1981; Randić, 1981). In this thesis, we show that NE, SS and L-glutamate are capable of modifying the electrical excitability of intraspinal sural primary afferent C- and A-fibers. Finally, our finding that the excitability of dorsal horn neurons to SP increases in 5-HT-depleated cats suggests a possible functional interaction between SP and 5-HT.

The observed excitatory and inhibitory actions of the above discussed neuroactive compounds could be the result of their action and/or interaction on either postsynaptic or presynaptic sites or both. However, in vivo studies are poorly suited for determining whether an action is postsynaptic or presynaptic. This problem has to be resolved in the in vitro slice preparation that allows a more precise determination of the site of action.

CCK-excitation:

Our results indicate that iontophoretically applied CCK-8 causes a moderate to strong excitation of about half of all tested neurons in laminae I-VII of the cat spinal cord and in the rat spinal cord slice preparation (see Section I). In the cat spinal cord, excitation was not limited to a single class of spinal units, but was observed in all three classes of neurons recognized in this area on the basis of their excitability by different kinds of cutaneous afferent input. These results are complementary to the similar observation of Phillis and Kirkpatrick (1979b) on frog spinal motoneurons and rat corticospinal neurons (Bunney et al., 1980; Phillis and Kirkpatrick, 1980), Dodd and Kelly (1981) on pyramidal neurons of the rat hippocampus, and Zetler (1979) on neurons of the myenteric plexus.

Our in vitro data with CCK-8 indicate that this peptide exerted its excitatory effect by a direct action on postsynaptic sites of the dorsal horn neurons, since the CCK-8 excitation was seen to persist in all tested units at a time when the synaptic transmission was blocked by superfusion of the slices with a Ca^{2+}-free, Mg^{2+}-high solution. This finding is in agreement with results of Phillis and Kirkpatrick (1979b) on the toad spinal cord showing that CCK causes direct postsynaptic TTX-insensitive depolarization of both dorsal and ventral roots and those of Dodd and Kelly (1981) demonstrating depolarization of CA1 rat hippocampal neurons bathed in low Ca^{2+}, high Mg^{2+} solution.

CCK-immunoreactivity has been detected in the small fibers having varicosities in all laminae of the spinal cord, as well as in the cell bodies in laminae II-V and in spinal ganglion (Larsson and Rehfeld, 1979;

Gibson et al., 1981). It has been shown that unilateral rhizotomy caused a significant depletion of CCK in the dorsal spinal horn (Yaksh et al., 1982). The latter finding suggests existence of the axoplasmatic transport of the peptide from the spinal ganglia to the axon terminals in the spinal cord. In addition, it has been shown that sciatic nerve stimulation released CCK, but only at stimulus intensities which activated Aδ- and C-fibers (Yaksh et al., 1982).

Our findings of a moderate to strong excitation of dorsal horn neurons produced by iontophoretically applied CCK-8 are consistent with an excitatory primary afferent function. However, presence of CCK in the both primary afferent fibers and in intrinsic dorsal horn neurons, makes it hard to believe that this is the only functional role of CCK in the spinal cord. Thus, the results obtained from the studies in the ventral tegmental area of the rat brain, where CCK is located in dopaminergic neurons and has an excitatory action (Bunney et al., 1980), led toward a proposal that CCK released together with dopamine may be a part of an inhibitory feedback system modulating dopamine release via action on dopamine autoreceptors (Hokfelt et al., 1980). Additional studies are obviously needed to shed more light on the possible site of CCK action and the role for CCK, either as a neurotransmitter or as a neuromodulator in the spinal dorsal horn.

VIP-excitation:

VIP applied by iontophoresis and/or pressure microinjection caused a strong excitation of more than 75% of all tested neurons in laminae I-VII of both the cat spinal cord and the rat spinal cord slice preparation.

In the intact cat spinal cord, the VIP-induced excitation similar to

that of CCK-8 was observed in all categories of neurons recognized in the spinal preparation of cats on the basis of their excitability by different kinds of cutaneous afferent input. Thus, our results are in agreement with the reported data about the potent excitatory action of VIP on frog spinal cord neurons and rat cerebral cortical neurons (Phillis et al., 1978; Phillis and Kirkpatrick, 1980), on CA1 neurons of the rat hippocampus (Dodd et al., 1979), on neurons of the myenteric plexus (Williams and North, 1979) and on neurons of the rat trigeminal nucleus caudalis (Salt and Hill, 1981).

Our results, obtained in the rat spinal cord slice preparation, showing that VIP depolarizes dorsal horn neurons and that this depolarization is associated with a decrease in neuronal membrane input resistance are in agreement with the observations made by Dodd et al. (1979) on CA1 neurons of the rat hippocampus. In addition, we have noted on several occasions that the depolarization of the dorsal horn neurons was of such a strength to cause inactivation of the spike generating mechanism, as shown also for the VIP action on CA1 neurons of the rat hippocampus (Dodd et al., 1979). This latter result could explain our frequent finding in the intact cat spinal cord namely that many cells responded with a brief excitation to VIP and that the excitation was associated with a significant decrease in the amplitude of the action potential and loss of a cell.

Diminished excitatory response of dorsal horn interneurons observed in our experiments with repeated applications of VIP at intervals less than 5 min probably represents a desensitization and is in agreement with the similar behavior to VIP of rat cortical neurons and frog motoneurons (Phillis et al., 1978) and neurons of the myenteric plexus (Williams and

North, 1979). A marked after-hyperpolarization of the dorsal horn neurons occasionally seen in our intracellular recordings in vitro offers a possible explanation for the desensitization and frequent loss of cells observed in the extracellular recordings from cat spinal cord neurons.

Immunohistochemical studies have shown that VIP is present in the spinal dorsal root ganglia (Lundberg et al., 1978), and that VIP positive fibers are concentrated in laminae I and II, although some are also found around the central canal (lamina X) and a few in lamina VII (Gibson et al., 1981). It appears that the spinal cord does not contain any VIP-positive neurons (Emson et al., 1979; Gibson et al., 1981). Unilateral rhizotomies of the lumbosacral dorsal roots but not the cervical hemisection reduced the levels of VIP in the dorsal horn significantly (Yaksh et al., 1982). In addition, electrical stimulation of the sciatic nerve evoked the release of VIP, but only at stimulus intensities which activated Aδ- and C-fibers (Yaksh et al., 1982). These findings are consistent with the view that all VIP in the spinal cord is of primary afferent origin.

Our finding of a strong excitation of dorsal horn neurons produced by locally applied VIP is consistent with an excitatory primary afferent function. However, in regard to the findings that VIP increases the intracellular concentration of cyclic adenosine monophosphate (Deschodt-Lanckman et al., 1977; Quik et al., 1979) and that the VIP-induced excitation of both spinal neurons and neurons in the nucleus caudalis of the trigeminal nerve (Salt and Hill, 1981) is of long duration, it is very likely that VIP has a regulatory or modulatory role as well. In addition, it has been shown that VIP induces a concentration-dependent glycogenolysis in mouse cortical slices (Magistretti et al., 1981). The latter finding

suggests a role of VIP in the regulation of energy metabolism within CNS.

In view of the observations that VIP is present in the thin primary afferent fibers in the spinal dorsal horn (Gibson et al., 1981) and that sciatic nerve stimulation evoked the release of VIP only when Aδ- and C-fibers were activated (Yaksh et al., 1982), the observed excitatory action on all classes of spinal cord neurons in our experiments is somewhat surprising. However, it must be remembered that there are populations of small fibers having slow conductance velocities, which transmit non-noxious mechanical and thermal sensory information (Iggo, 1960, 1969).

Augmentation of the SP excitation in the 5-HT-depleted spinal cord:

The coexistence of SP and 5-HT in the same neurons of the brain stem raphe system and in the same nerve endings in the spinal cord has been demonstrated (Hökfelt et al., 1977, 1978; Chan-Palay et al., 1978; Björklund et al., 1979; Chan-Palay, 1979; Pelletier et al., 1981). Since the physiological significance of coexistence of SP and 5-HT within the same spinal cord nerve endings is unclear, we approached this question by examining whether the excitability of functionally identified dorsal horn neurons in response to iontophoretic application of SP is affected by depletion of central 5-HT stores with p-CPA. We found that the excitability of dorsal horn neurons to SP increases in 5-HT-depleted cats.

In the light of earlier studies on the functional interaction between SP and 5-HT (Rivot et al., 1980; Davies and Roberts, 1981), the observed increase in the excitability of dorsal horn neurons to iontophoretically applied SP, may have been a result of a reduction of the tonic descending inhibition by the p-CPA-induced 5-HT depletion. Although more recent

findings challenge 5-HT involvement in tonically active inhibitory descending systems in the cat spinal cord (Soja and Sinclair, 1980; Hall et al., 1981). Namely, in the p-CPA-pretreated rat spinal cord, Rivot et al. (1980) found that, in addition to reduction of descending inhibitory influence on spinal dorsa horn interneurons from NRM stimulation, about 35% of WDR neurons were excited by NRM stimulation. They suggested that this increased excitability of spinal cells could be a consequence of SP released by NRM stimulation. However, they were not able to show whether p-CPA specifically unmasked the SP effects or other excitatory descending pathways. Recently, Davies and Roberts (1981) reported that the responses of dorsal horn neurons to iontophoretically applied SP were selectively attenuated by iontophoretically applied 5-HT and suggested that the probable site of this interaction is postsynaptically on dorsal horn interneurons where 5-HT might be acting either upon the SP receptor or the receptor-excitation coupling mechanism. Using a synaptosomal preparation obtained from the rat ventral lumbar spinal cord, Mitchell and Fleetwood-Walker provided a direct demonstration of SP and 5-HT interaction at the receptor level. They found that a reduction in the stimulus-evoked release of 5-HT produced by activation of 5-HT autoreceptors was clearly antagonized by SP.

From the above discussion, it is evident that further investigations are needed in order to increase knowledge about the physiological significance of coexistence of SP and 5-HT within the terminals of the spinal dorsal and ventral horns.

Presynaptic effects of NE, SS and L-glutamate:

NE, SS and L-glutamate are present in the superficial laminae of the mammalian spinal dorsal horn, the same area where primary afferent fibers of fine diameter (Aδ and C) are shown to terminate (Light and Perl, 1979; Ralston and Ralston, 1979). It is a prevalent view held today that the effects of locally applied NE and L-glutamate on dorsal horn interneurons are exerted postsynaptically (Curtis et al., 1960; Engberg and Ryall, 1966; Engberg et al., 1979) although a presynaptic site of action has also been suggested (Curtis and Ryall, 1966; Evans, 1978; Phillis and Kirkpatrick, 1979a). It is not clear, however, whether SS acts presynaptically or postsynaptically or both (Murase et al., 1982).

Before discussing our data demonstrating that NE, SS and L-glutamate modify the electrical excitability of primary afferent fibers, it is relevant to describe the present concepts about ways in which the amount of transmitter released from the terminals of primary afferent fibers may be modulated.

It is now well-established fact that primary afferent terminal membrane potential relates to the amount of the excitatory transmitter released (Schmidt, 1971). The most extensively investigated phenomena are primary afferent depolarization (PAD) and primary afferent hyperpolarization (PAH). Thus, PAD leads to a decrease in the amount of excitatory transmitter released from the terminals (Eccles et al., 1963a; Schmidt, 1971). Although PAH is considered to underlie presynaptic facilitation (Eccles, 1964) and would theoretically result in an increase in the amount of excitatory transmitter released, another view is also expressed by Sastry (1979a), namely that PAH may cause reduction of transmitter release by retarding

the invasion of the terminal by the action potential. In this respect, it is of interest that at the crustacean neuromuscular junction presynaptic inhibition of excitatory transmission is accompanied by a hyperpolarization of the excitatory axon (Kawai and Niwa, 1978). Another possible mechanism of presynaptic inhibition considered is that a conductance increase blocks the conduction of action potential through fine terminal processes, thereby preventing the action potential from fully invading the output sites (Atwood, 1976). Recently, Dunlap and Fischbach (1978, 1979) demonstrated that several putative neurotransmitters, among these NE and SS, decrease the calcium component of cell body action potentials of cultured chick sensory neurons. In addition, NE and SS inhibited K^+-evoked release of SP (Fischbach et al., 1981). These findings are of considerable importance and if the same phenomenon described for the somatic action potentials of dorsal root ganglia happens to occur at the presynaptic terminal level it would be a novel mechanism for presynaptic modulation of transmitter release.

The technique of testing the electrical excitability changes of the single primary afferent fibers as used in our work is presently the method of choice in the analysis of presynaptic site of action of a potential neurotransmitter or a neuromodulator substance. From excitability testing of large A-fibers, it is thought that a decrease in threshold of their central terminals corresponds to a PAD (Eccles et al., 1963a). However, several mechanism for the threshold increase have been suggested. One being that it reflects PAH (Carstens et al., 1979). Alternatively, an increase in threshold for antidromic activation of a fiber may be expected if a membrane conductance is increased by a drug. The latter would create

a current shunt, which would necessitate a greater stimulus current density to antidromically excite the fiber (Carstens et al., 1981).

The technique used in our studies for testing the electrical excitability of the intraspinal single sural primary afferent fibers during local application (iontophoresis and/or pressure microinjection) of NE, SS and L-glutamate besides the proven advantages has also some limitations. Thus, the extent to which actions of the test substances are confined to the nerve terminals, or involve the preterminal axons as well, cannot be determined by this technique. Although the local application of chemicals offers significant advantages as compared to the systemic route, iontophoretic application of a chemical may produce changes in extracellular resistance due to ion fluxes and alterations in extracellular volume; the latter in turn may change the threshold for antidromic activation (Krnjević et al., 1980; Segal and Gutnick, 1980). To minimize this artifact we used numerous current controls (ejection of Na^+, Cl^-, acetate ions or tartrate ions) and also a current neutralization procedure. The latter technique rules out the possibility that the polarization level of fibers is changed after their conductance has been altered by the drug.

Our results indicate that a local administration of NE into the superficial layers of the spinal dorsal horn reduces the electrical excitability of about 68% of sural afferent C-fibers and more than 85% of A-fibers tested. In addition, about 5% of C-fibers showed reproducible increase in excitability, 8% of C-fibers showed dose-related biphasic effects, and the rest were unaffected. The implication of these results is that a decrease in excitability produced by locally applied NE in the superficial layers of the spinal dorsal horn is relatively non-selective.

Contrary to our data showing an indiscriminate increase in the threshold of primary afferent fibers, Hentall and Fields (1979) demonstrated that the intraspinal thresholds of some polymodal nociceptors and high threshold mechanoreceptors with afferent C-fibers were raised during electrical stimulation of the brain stem areas containing monoamines. Carstens and Zimmermann (1981) confirmed that stimulation in the midbrain induces an increase in the threshold of C-fibers, although the effect was not consistently observed. In addition, Hodge and his collaborators (Apkarian et al., 1981; Hodge et al., 1981) have shown that the electrical stimulation of the locus coeruleus causes NE-dependent depolarization of dorsal roots and inhibition of spinal dorsal horn interneurons.

Dunlap and Fischbach (1978) have demonstrated that application of NE in doses that did not alter resting potential or conductance, decreased the influx of Ca^{2+} across the activated soma membrane of cultured dorsal root ganglion cells. In addition, Fischbach et al., (1981) have found that NE inhibited K^{+}-evoked release of SP from cultured chick sensory neurons. These findings, if extrapolated to the primary afferent terminals, raise the possibility that NE may modify transmitter release from primary afferent terminals by directly affecting Ca^{2+} influx.

In relation to the recent reports suggesting that α-adrenergic receptors play a major role in mediating the effects of NE in the spinal cord (Dunlap and Fischbach, 1978; Kawasaki et al., 1978; Kuraishi et al., 1979a,b; Yaksh, 1979; Reddy and Yaksh, 1980; Reddy et al., 1980), we have examined the effects of several sympatomimetics (clonidine, dopamine, isoproterenol and phentolamine) and α-adrenergic antagoinists (phentolamine and yohimbine) on the excitability of sural afferents and the NE-produced

threshold change in C- and A-fibers, respectively. We have found that both α- and β-receptor agonists cause a reduction of the excitability of C- and A-fibers, clonidine being the most potent in this respect and isoproterenol the least potent. These results suggest that NE may be acting at a population of α-receptors, although involvement of β-receptors cannot be excluded because of the qualitatively similar effect of isoproterenol. It has not been possible to produce any convincing evidence of a blockade of the NE-induced threshold increase in C-fibers, either by iontophoretically applied phentolamine and yohimbine or by i.v. applied yohimbine. Our inability to block the NE-induced change in the threshold in C-fibers does not mean that α-receptors are not involved, because antagonists by themselves, had strong effects on the excitability of primary afferent fibers.

It cannot be determined with our method whether NE acts directly at the intraspinal primary afferent fibers or indirectly via dorsal horn interneurons possibly mediating presynaptic excitability changes at the C-fibers. Because of the evidence for the involvement of adrenergic mechanisms in modulation of spinal processing of nociceptive information (Belcher et al., 1978; Headley et al., 1978), and the observation that enkephalin induces the decrease in excitability of primary afferent fibers (Sastry, 1979a), it was of interest to test effects of naloxone on the depressant action of NE on the excitability of C-fibers. As our data show, naloxone had no antagonistic effect on the threshold increase produced by NE in C-fibers. These results exclude involvement of enkephalin-containing interneurons in the NE-induced depression of C-fibers excitability. Morphological data, however, do not suggest direct synaptic contacts, i.e. existence of axo-axonic synapses, between descending NE-containing fibers

and primary afferents (Ruda et al., 1979).

Our results showed that a local administration of SS into the superficial layer of the spinal dorsal horn produced an increase in excitability of about half of the C- and Aδ-fibers tested. This effect appeared to be dose-related and reversible. In addition, a reproducible threshold increase was observed in about 16% of the C-fibers tested.

From the electrical excitability testing of the large diameter A-fibers, it is known that a decrease in threshold corresponds to a PAD (Koketsu, 1956; Eccles and Krnjević, 1959; Eccles et al., 1963a; Hodge, 1972). Therefore, PAD may be a mechanism responsible for the increase in excitability of C- and Aδ-fibers produced by SS in our experiments. However, Fischbach and his collaborators proposed an alternative mechanism for presynaptic inhibitory action of SS (Dunlap and Fischbach, 1978; Fischbach et al., 1981). In the cell bodies of embryonic chick dorsal root ganglion neurons maintained in cell culture, it was found that SS decreases the inward Ca^{2+} current (Dunlap and Fischbach, 1978) and inhibits the K^{+}-evoked release of SP (Fischbach et al., 1981). If SS was to act in a similar manner at the level of sensory nerve terminals, this would be a novel mechanism for the presynaptic inhibition.

It cannot be determined with our method whether SS acts directly and/or indirectly via modulation of activity of dorsal horn interneurons, possibly mediating presynaptic excitability changes at the primary afferent fibers. Morphological studies have demonstrated the presence of a small population of axo-axonic synapses in the superficial laminae of the dorsal horn having C-fibers as postsynaptic elements (Ralston, 1979; Zhu et al., 1981). Although the number of these synaptic contacts is small, their complexity

and presence of different types of synaptic vesicles within the pre- and postsynaptic elements suggest that this type of contact may play an important role in modulation of sensory information coming into the dorsal horn (Zhu et al., 1981).

The SS-induced decrease in the threshold for antidromic activation of primary afferent C- and Aδ-fibers may explain in part the depressant action of iontophoretically applied SS on dorsal horn interneurons (Randić and Miletić, 1978).

Iontophoretically applied L-glutamate into the superficial layers of the spinal dorsal horn increases excitability of about half of the single intraspinal sural afferent C- and A-fibers. The effect appeared to be dose-related and reversible. The depolarizing action of L-glutamate on the primary afferent fibers shown in our experiments is in agreement with the data obtained in the toad (Schmidt, 1963) and feline (Eccles et al., 1963b) spinal cord. Using Wall's method of excitability testing, Curtis and Ryall (1966) found that L-glutamate reversibly increased the electrical excitability of single large cutaneous and muscle afferents.

Although we are not able to determine with our method whether L-glutamate acts directly at the intraspinal afferent fibers and/or indirectly via modulation of activity of dorsal horn interneurons, our inability to antagonize L-glutamate increase in excitability with bicuculline eliminates the possibility that GABA-containing interneurons are involved in L-glutamate effect.

Since De Groat et al. (1972) demonstrated that L-glutamate does not depolarize dorsal root ganglion cells, it was proposed that receptors for L-glutamate are confined exclusively to the central terminals of primary

afferent neurons (Curtis et al., 1977; Evans, 1978). However, Evans (1980) recently presented results which suggest that L-glutamate effects on the terminals of primary afferent neurons in the frog spinal cord may be produced indirectly through the release of K ions from glutamate-excited dorsal horn neurons. Our findings showed that only about 50% of C- and Aδ-fibers were affected by L-glutamate. In comparison, L-glutamate-induced an increase in excitability of all Aβ-fibers which suggests the possibility of specific receptors involvement. An increase in K^+ as the causative factor must also be given serious consideration.

In conclusion, the results presented in this thesis suggest that: (1) CCK-8 and VIP have excitatory actions on all categories of neurons recognized in the spinal preparations of cats on the basis of their excitability by different kinds of cutaneous afferent input; (2) excitability of functionally identified dorsal horn neurons to iontophoretically applied SP increased in 5-HT-depleated cats; (3) NE, SS and L-glutamate are capable of modifying the electrical excitability of intraspinal sural primary afferent C- and A-fibers.

SUMMARY

1. The purpose of this project was to study the actions of neuroactive peptides such as CCK, SS, SP and VIP as well as L-glutamate and biogenic amines (5-HT and NE) on the excitability of functionally identified dorsal horn neurons and single cutaneous primary afferent fibers in the cat spinal cord *in vivo*.
2. Neuronal activity of functionally identified spinal dorsal horn neurons was monitored extracellularly while the CCK-8, VIP and SP were applied locally by iontophoresis and/or pressure microinjection.
3. An excitability testing procedure of single primary afferent C- and A-fibers was used to study possible presynaptic actions of NE, SS and L-glutamate applied locally at the intraspinal sites of minimal threshold for their antidromic activation.
4. CCK-8, applied iontophoretically, caused a moderate to strong excitation of about half of all tested neurons in laminae I-VII of both the cat intact spinal cord and the rat *in vitro* spinal cord slice preparation.
5. In the intact cat spinal cord, the excitation was not limited to a single population of neurons but was observed in all categories of units recognized in spinal preparations of cats in this area on the basis of their excitability by different kinds of cutaneous afferent input.
6. CCK action appeared to be a direct one exerted on postsynaptic sites of the dorsal horn neurons, since it persisted when the spinal cord slices were perfused with Ca^{2+}-free, Mg^{2+}-high Krebs solution.

7. These results are consistent with the possibility that CCK-8 acts on postsynaptic sites in the dorsal horn of the spinal cord as a neurotransmitter or neuromodulator.
8. VIP, applied by iontophoresis and/or pressure microinjection, caused a strong excitation of more than 75% of all dorsal horn neurons tested in laminae I-VII of both cat intact spinal cord and the rat spinal cord slice preparation.
9. In the intact cat spinal cord, the excitation was not limited to a single population of neurons but was observed in all categories of units recognized in spinal preparations of cats in this area on the basis of their excitability by different kinds of cutaneous input. The desensitization of the responses with repeated applications of VIP was observed.
10. In the rat spinal cord slice preparation, VIP-induced depolarization was associated with a decrease in neuronal membrane input resistance.
11. These results are consistent with the possibility that VIP may have a physiological role in synaptic function, either as a transmitter or as a modulator.
12. Iontophoretic application of synthetic SP caused moderate to strong excitation of about 80% of cat spinal neurons activated either by noxious mechanical and/or thermal stimuli and about 31% of neurons activated by innocuous stimuli.
13. Three days after a single dose of p-CPA (350 mg/kg i.p.) there was a marked decrease in the 5-HT content of the lumbosacral spinal cord while the levels of SP, CCK, VIP and NT were not consistently changed.

14. In the p-CPA-pretreated cats, iontophoretically applied SP caused excitation of all tested neurons.

15. These results indicate that the excitability of dorsal horn neurons to SP increases in 5-HT-depleted cats.

16. NE, applied by iontophoresis and/or pressure microinjection, produced dose-related increases in threshold for antidromic activation of about 68% of sural afferent C-fibers and 90% of A-fibers tested. In addition, about 5% of tested C-fibers showed reproducible increase in excitability, 8% dose-related biphasic effects, and the rest (19%) were unaffected.

17. Both α- and β-adrenergic agonists cause a reduction of the excitability of C- and A-fibers, clonidine being the most potent in this respect and isoproterenol the least potent.

18. These results suggested that activation of catecholaminergic descending pathways may exert a modulating influence over the processing of sensory information carried by cutaneous primary afferent C- and A-fibers into the spinal cord.

19. Iontophoretically applied SS into the superficial layers of the spinal dorsal horn increased the electrical excitability of about half of sural primary afferent C- and A-fibers. In addition, about 16% of C-fibers tested with SS showed reproducible decrease in excitability. The effects appeared to be dose-related and reversible.

20. Iontophoretically applied L-glutamate into the superficial layers of the spinal dorsal horn increased excitability of about half of single intraspinal sural afferent C- and Aδ-fibers and all Aβ-fibers. The effects appeared to be dose-related and reversible.

21. Effect of L-glutamate on the excitability of primary afferent fibers was not antagonized by iontophoretic application of bicuculline.

22. These results suggested that in addition to previously shown post-synaptic effects, SS and L-glutamate increase excitability of intraspinal cutaneous primary afferent C- and A-fibers.

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