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Morphological and functional studies of the contribution of the substance P receptor to nociceptive processing

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Morphological and Functional Studies of the Contribution of the Substance P Receptor to Nociceptive Processing

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Jessica Brown

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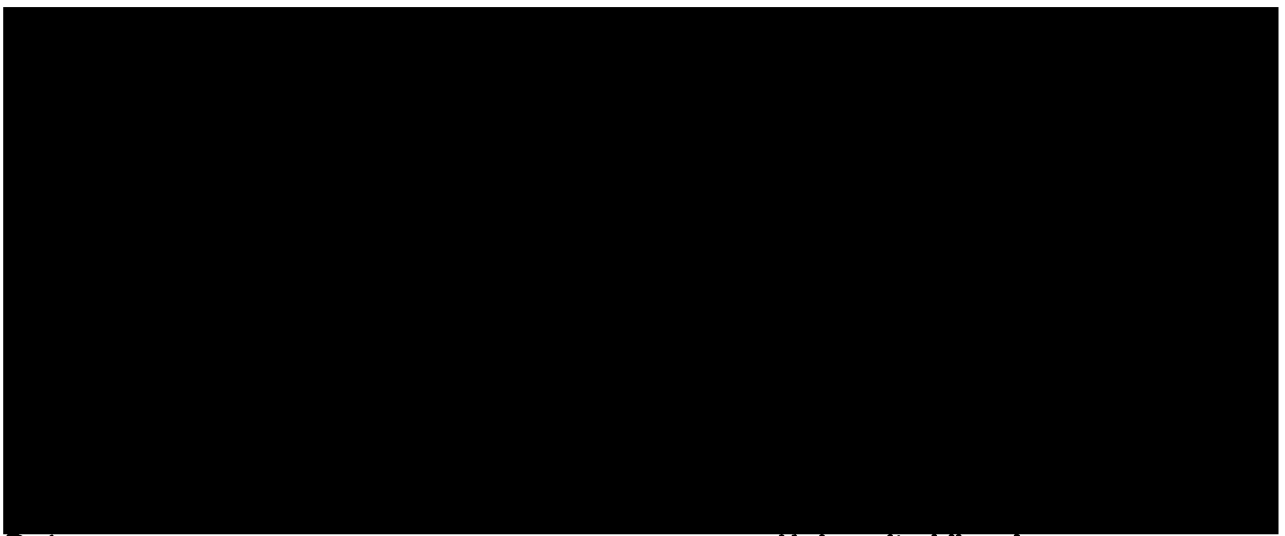

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Morphological and Functional Studies of the Contribution of the Substance P Receptor to Nociceptive Processing

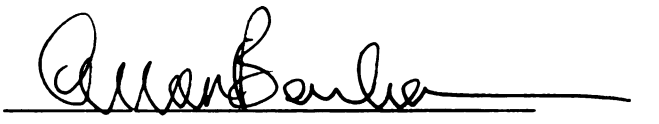
Jessica L. Brown

ABSTRACT

Although there is considerable evidence that primary afferent-derived substance P contributes to the transmission of nociceptive messages especially at the level of the spinal cord, the population of neurons that respond to substance P has not been completely characterized. To address this question, we used an antibody directed against the C-terminal portion of the rat substance P receptor to examine the cellular distribution in neurons of the rat brain and spinal cord. The substance P receptor (SPR) appeared to be exclusively expressed by neurons; in fact, the substance P receptor decorates the somatic and dendritic surfaces of the neurons, producing Golgi-like images. Electron microscopic results revealed that approximately 70% of the surface membrane of immunoreactive neurons is laden with substance P receptor.

Light microscopic results have revealed morphologically distinct populations of neurons that express the SPR throughout the rat brain and spinal cord. Within the dorsal horn of the spinal cord, we identified morphologically distinct populations of neurons that express the SPR in lamina I and the lamina V, areas that contain noci-responsive neurons. Retrograde tracer studies have shown that a large percentage of SPR lamina I neurons project to supraspinal sites, specifically the parabrachial nucleus and the nucleus tractus solitarius, both of which have been implicated in nociceptive processing. Double-labeling studies, using Fos as a marker of activity, have shown that a large proportion of SPR neurons in the dorsal horn of the spinal cord express Fos after noxious stimulation.

These findings indicate that a large proportion of SPR spinal cord neurons are activated after noxious stimulation and comprise an important component of nociceptive processing in the dorsal horn of the spinal cord. Results demonstrate the spinal cord circuitry through which substance P exerts its effects in nociceptive processing.



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CHAPTER I

Introduction

Substance P: isolation and characterization

The undecapeptide substance P (SP) is an important signalling molecule that has been implicated in several different neurobiologic systems. In the early history of SP, von Euler and Gaddum (1931) discovered SP through its effects on circulation and the gastrointestinal tract. During this period, more quantitative results required purification procedures, after which the substance became generically referred to as "preparation P". This ultimately gave rise to the name Substance P (von Euler, 1977). Early studies, which concentrated on the effects of SP on peripheral organs, established that SP induced smooth muscle contraction in the intestines, vasodilation, and was a potent sialogogic substance. These pharmacologic bioassays set the foundation for future characterization of SP and its corresponding endogenous receptor within the nervous system.

The actual purification of SP resulted inadvertently from attempts to isolate the corticotropin-releasing factor; during these studies Leeman and Hammerschlag isolated a peptide with sialogogic activity from bovine hypothalamus (Leeman and Hammerschlag, 1967). Upon purification, the pure substance had biological properties similar to those described for substance P (Chang and Leeman, 1970). Sequence analysis of the purified compound determined the amino acid composition to be Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met (Chang et al., 1971). Ultimately, Studer and associates isolated SP from horse intestine, purified it according to the Chang and Leeman procedure, and finally sequenced it (Pernow, 1983). The result was a peptide with the identical amino acid composition previously obtained by Chang et al. These two parallel experiments confirmed that the sialogogic peptide was indeed substance P; and furthermore that brain and intestinal SP were the same.

The Tachykinin Family

SP is but one peptide in the family of tachykinins, so called because of their ability to induce rapid contractions of smooth muscle. Erspamer (1981) initially described the structure and relationship among the tachykinin peptides; they all share a common C-terminal sequence Phe-X-Gly-Leu-Met. Although a number of the earlier analyses were based upon non-mammalian derived tachykinins, within the past decade recent molecular biology and biochemistry studies have greatly expanded our knowledge of the mammalian tachykinin peptides.

The mammalian tachykinin peptides include substance P, neurokinin A, neuropeptide K, neuropeptide gamma, and neurokinin B. These peptides are the products of two evolutionarily related genes: the neurokinin A (NKA) gene and the neurokinin B (NKB) gene. Neurokinin B is the only known tachykinin peptide to be produced from the NKB gene; the remaining tachykinin peptides are different splice variants of the SP/NKA gene.

Pharmacological activity of the tachykinins shows many similarities in the different peripheral organ preparations, however, the relative potency of the tachykinins differ. Substance P is the most potent vasodilator; physalaemin is a more potent sialogogic substance. Based upon these pharmacologic differences, the family of peptides is typically subdivided into three primary groups, SP, NKA, NKB based on the rank order potencies of the ligands at the three characterized neurokinin receptors (Helke et al., 1990).

SP Receptor

Parallel to the rank order potencies of the tachykinin ligands, three classes of neurokinin receptors have been classified. The family of tachykinin peptides interact with known receptor subtypes: neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3). Substance P displays the highest affinity for NK-1; while

neurokinin A and neurokinin B bind preferentially to NK-2 and NK-3, respectively. In the absence of selective antagonists at the time of classification, receptor characterization was wholely based on the rank order potencies of the tachykinin peptides. In other words, at the NK-1 site, the order of agonist potency is SP>NKA>NKB; for the NK-2 site, the order of agonist potency is NKA>NKB>SP; finally at the NK-3 site, the order is NKB>NKA>SP. Cloning of the three neurokinin receptors has confirmed their existence. It should be noted, however, that the tachykinin peptides, as a whole, can produce physiological actions at nanomolar concentrations at the different receptors. Further examination of the neurokinin receptors with the newly developed tachykinin antagonists suggests the presence of different receptor subtypes (Burcher et al., 1991).

Mechanisms of the SP Receptor

Sequencing of the tachykinin receptors has revealed structural and functional similarities to the G-protein linked superfamily of receptors. Analysis of the SPR sequence revealed a seven transmembrane topology, which is the most common feature of the G-protein linked receptors. Phosphatidyl inositol turnover has also been linked to the activation of the tachykinin receptors, presumably through the activation of the phospholipase C cascade (Krause et al., 1991).

Part II: SP Implications for the Central Nervous System

Not long after the initial demonstration of SP, studies with brain extracts indicated that nervous tissue contained relatively high levels of the active substance "P" (von Euler, 1977). Early studies showed that the brains from various species contained SP, and that species with less differentiated brains contained higher densities of SP (Pernow, 1983). In general, the subcortical regions of the brain showed higher levels of SP than did the cortex.

Furthermore, administration of semi-purified preparations of SP elicited a whole host of effects in the central nervous system (CNS), among which was antagonism of morphine analgesia (Stern and Hukovic, 1960). Intracerebroventricular injection of partially purified SP induced a long-lasting stimulation of respiration as well as an inhibition of "spontaneity", i.e. stupor (von Euler and Pernow, 1956). Local administration of SP preparations into the cortex produced increases in cortical activity (Pernow, 1983). Although these early findings were conducted with an unpurified preparation of SP, they were suggestive of the potential actions of SP in the CNS.

After the purification and sequencing of SP, it became possible to develop antibodies against SP, which could then be used to better localize the SP distribution throughout the CNS. A large number of radioimmunological and immunohistochemical studies have examined the SP distribution in nervous tissue. Although several of the early SP antibodies were not tested for cross-reactivity with the related tachykinin peptides, because these peptides had not yet been isolated, confirmation with more current antibodies has provided ample support for the diffuse distribution of SP throughout the CNS.

Brain

Several comprehensive mapping studies have demonstrated common regions of dense SP immunoreactivity throughout the rat CNS (Cuello and Kanazawa, 1978; Hökfelt et al., 1975a; Ljungdahl et al., 1978; Shults et al., 1984). High densities of SP immunoreactivity are found in both the central and medial amygdala, the septal complex, and the caudate nucleus; the cerebral cortex is largely devoid of immunoreactivity. Additionally, nuclei of the hypothalamus, the habenula complex, the interpeduncular nucleus, the substantia nigra, the dorsal tegmental nucleus, the locus coeruleus, and the parabrachial nucleus also showed high levels of SP immunoreactivity. *In situ* hybridization of the SP messenger RNA (Kiyama et al., 1993) have helped confirm the localization of the SP expressing neurons.

Lesion studies have also helped determine the connectivity of SP neurons within the CNS. For example, it has been established that portions of the SP immunoreactivity in the septal complex and interpeduncular nucleus originate from the lateral dorsal tegmental nucleus and the habenula complex, respectively (Cuello et al., 1982; Hong et al., 1976; Sakanaka et al., 1981). One of the most well examined SP pathways is the projection of SP from the striatum to the substantia nigra (Gerfen, 1992; Kanazawa et al., 1977; Mroz et al., 1977).

Despite the comprehensive anatomical studies on SP localization, the functions attributed to SP in different regions of the brain is rather limited. Evidence has supported a contribution of SP in defensive rage behaviors elicited through the medial amygdala (Shaikh et al., 1993). Injection of SP into specific brainstem sites has provided evidence for a contribution to the modulation of nociceptive messages (Yeomans and Proudfit, 1992). In the striatum, there is evidence that the SP output to the substantia nigra is an important component in modulating the electrophysiological activity of the substantia nigra, which in turn modulates thalamic activity (Gerfen, 1992).

Although a more accurate means of predicting areas of SP functioning in the brain might be through the localization of the SP receptor, early studies of the SPR distribution in the CNS have resulted in considerable evidence for mismatches between receptor and peptide (Shults, et al., 1984). In fact, radioligand binding studies indicate that there is no consistent relationship between the distributions of SP and its endogenous receptor. There are regions in the brain with both SP and SPR, with SP but little SPR, and with SPR but little SP. Thus, localization of the SP binding did not fully explain the role of SP in the brain. Rather it generated many more questions.

Spinal Cord

The contribution of SP in spinal cord and trigeminal nucleus caudalis processing has been most extensively studied. These studies provided compelling evidence of SP's involvement in nociceptive transmission. The highest density of SP in the spinal cord is found in the superficial laminae of the dorsal horn (I and II). The origin of SP in the superficial spinal cord was demonstrated in a series of ligation and transection studies. When the dorsal roots of the spinal cord were sectioned or ligated, the levels of SP immunoreactivity in dorsal horn were dramatically reduced, while SP immunoreactivity accumulated on the side of the ganglion (Hokfelt et al., 1975b). These data suggest that SP is synthesized in the dorsal root ganglion neurons and then transported to the primary afferent terminals for release into the superficial dorsal horn. The fact that SP expression in the sensory ganglion was localized primarily to the population of small neurons with unmyelinated, fine diameter axons was fundamental to understanding SP's involvement in nociceptive processing. These neurons are known to respond to high intensity, nociceptive stimulation (Barber et al., 1979; Hokfelt et al., 1975a; Ljungdahl, et al., 1978). Moreover, when unmyelinated C-fibers are selectively destroyed by capsaicin treatment in neonatal rats, the SP content in the

dorsal horn is significantly reduced and the thresholds for chemical and mechanical nociception are increased (Hayes and Tyers, 1980). Furthermore, behavioral studies have demonstrated that intrathecal administration of SP at the lumbar level evokes a morphine sensitive pain behavior that consists of scratching and biting at the hind limbs (Frenk et al., 1988). While it is still controversial whether the responses to intrathecal SP are representative of pain, selective NK-1 antagonists can block these evoked behaviors.

Other studies have examined the release of SP in the spinal cord and the electrophysiological responses of spinal cord neurons to SP microinjection or iontophoresis. Both noxious mechanical stimulation of the skin and electrical stimulation of peripheral nerves at C-fiber strength evokes the release of SP in the spinal cord (Duggan et al., 1988). Other stimulation paradigms that result in tissue damage and inflammation also result in SP release in the superficial dorsal horn. Electrophysiological recordings of nociresponsive neurons in the dorsal horn exhibit prolonged depolarization in response to SP application (Henry, 1976; Salter and Henry, 1991); this response is inhibited by selective NK-1 antagonists (Yashpal et al., 1993). It is still not entirely clear whether SP exerts actions on non-nociresponsive neurons.

Somewhat consistent with the distribution of SP, localization studies show substance P binding sites primarily within the superficial laminae of the dorsal horn of the spinal cord. However, this finding is controversial in light of the majority of electrophysiological studies which show SP-evoked responses in nociresponsive neurons located in deeper laminae of the dorsal horn, i.e. where SP terminals are much less abundant. This spinal cord anomaly is a type of functional receptor mismatch, an extension of the previously cited SP and SPR mismatch observed in the brain.

One explanation for these discrepancies in SPR localization is the limitations of the radioligand binding and *in situ* hybridization techniques that have been used to

localize the SPR. In situ hybridization labels cell bodies; the absence of dendritic labelling makes it impossible to identify the morphology of labelled neurons (Kiyama, et al., 1993). Radioligand binding studies label both cell bodies and dendrites, but the resolution is not adequate to identify individual neurons (Charlton and Helke, 1985; Helke et al., 1986; Yashpal et al., 1990). Given these issues of resolution surrounding the localization of the SPR, it is questionable whether either in situ hybridization or receptor binding provides a complete analysis of the SPR distribution.

With an antibody directed against the carboxyl end of the rat substance P receptor, we can now address many of the issues surrounding the localization of the SPR throughout the CNS. In addition, we can also begin to address functional questions about the population of neurons that express the SPR and which are thus likely to respond to its release after noxious, pain producing stimulation.

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CHAPTER II

Synaptic relationship between substance P and the substance P receptor: Light and electron microscopic characterization of the mismatch between neuropeptides and their receptors

Synaptic relationship between substance P and the substance P receptor: Light and electron microscopic characterization of the mismatch between neuropeptides and their receptors

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ABSTRACT

Light microscopic studies have demonstrated significant mismatches in the location of neuropeptides and their respective binding sites in the CNS. In the present study we used an antiserum raised against a synthetic peptide corresponding to the carboxyl-terminal tail of the Substance P receptor (SPR) to further explore the relationship between a neuropeptide and its receptor. Light microscopy revealed an excellent correlation between the patterns of SPR immunoreactivity and of ^{125}I-SP receptor binding sites in the CNS. The SPR appeared to be exclusively expressed by neurons; in fact, the SPR decorates the somatic and dendritic surface of neurons, producing Golgi-like images. Electron microscopic analysis in cortex, striatum and spinal cord revealed that approximately 70% of the surface membrane of immunoreactive neurons is SPR-laden. Simultaneous EM labelling of SP and SPR demonstrated significant mismatch at the synaptic level. Although some SP terminals contacted SPR-immunoreactive membrane, no more than 15% of the SPR-laden membrane apposed synaptic terminals. These results suggest that in contrast to more "classical" central and peripheral nervous system synapses, wherein the receptor immediately apposes the site of neurotransmitter storage and release, much of the surface of SPR-expressing neurons can be targeted by SP that diffuses a considerable distance from its site of release.

At a "classical" neuronal synapse, neurotransmitter is released from presynaptic vesicles by exocytosis, crosses the synaptic cleft and binds to receptors located postsynaptically. Implicit in this characterization is that neurotransmitter release sites at these synapses are closely apposed to the targetted receptor; neurotransmitter and receptor are separated only by the synaptic cleft and density between pre- and postsynaptic elements (Fagg and Matus, 1984; Petralia and Wenthold, 1992; Triller et al., 1985; Van den Pol and Gorcs, 1988) Examples includes glycinergic (Triller et al., 1985; Van den Pol and Gorcs, 1988) and some glutamatergic synapses (Fagg and Matus, 1984; Petralia and Wenthold, 1992) in the CNS and cholinergic synapses in sympathetic ganglia (Jacobs et al., 1986) and at the adult neuromuscular junction (Fertuck and Salpeter, 1974). On the other hand, some GABA receptors in the cerebellum are located at sites away from GABA-containing synapses, raising the possibility that GABA can act upon targets distant from its site of release, in a "non-synaptic" fashion. The fact that postsynaptic densities are absent at some CNS monoaminergic synapses (Beaudet and Descarries, 1978) suggests that noradrenaline and serotonin have a similar action.

Peptide neurotransmitters, which often colocalize with more classical neurotransmitters (Hokfelt et al., 1980) may also act in a diffuse, non-synaptic manner. Thus, for example: 1) there are significant mismatches between the distribution of peptides and their respective binding sites (Herkenham, 1987; Mantyh et al., 1984a; Mantyh et al., 1984b); 2) peptide neurotransmitters can diffuse away from their site of release (Duggan et al., 1990; Duggan et al., 1992), and can even be recovered in spinal cord CSF (Yaksh et al., 1980); 3) binding sites for mu (Hamel and Beaudet, 1984) and delta (Pasquini et al., 1992) opioid peptides and for neurotensin (Beaudet and Woulfe, 1985) rarely overlap synaptic densities; 4) dense core vesicles that contain neuropeptides are usually located away from the synaptic density, which is the presumed site of release of classical neurotransmitters (De Biasi and Rustioni, 1988; Merighi et al., 1991); 5) the locus of

exocytosis of dense core vesicles can, in fact, be distant from the synaptic junction (density) (Buma and Roubos, 1986; Zhu et al., 1986) In the present report we use an antiserum directed against the substance P receptor (SPR), which corresponds to the NK-1 subtype of tachykinin receptors (Mantyh et al., 1989a), and demonstrate that there is indeed significant mismatch at the synaptic level between peptide and peptide receptor. Furthermore, we demonstrate that the SPR receptor decorates a large proportion of the somatic and dendritic surface of subpopulations of CNS neurons, indicating that much of the neuronal surface is a potential target of peptide neurotransmitter.

METHODS

The studies were performed on male, Sprague-Dawley rats (240-260gm) that were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused through the ascending aorta with 100 ml 0.1M phosphate-buffered saline (PBS; pH 7.4) followed by an 0.1M phosphate-buffered fixative solution containing either 4.0% paraformaldehyde (for light microscopy) or 2.0% glutaraldehyde, 0.5% formaldehyde and 0.2% picric acid (for both light and electron microscopy), according to the protocol of Llewellyn-Smith et al (1992). After the perfusion the brain and spinal cord were removed and postfixed in the same solution for 2 - 4 hours.

The autoradiograms of ^{125}I-SP binding were generated as previously described (Mantyh, et al., 1989a). For immunocytochemistry, we used an anti-SPR antibody that was raised against a 15 amino acid peptide sequence (SPR393-407) at the carboxyl-terminus of the rat substance P receptor (Vigna et al., 1993) The immunogen consisted of synthetic peptide conjugated to bovine thyroglobulin using glutaraldehyde. The antiserum used in this study (#11884-5) recognized a protein band of 80-90kD on Western blots of membranes prepared from cells transfected with the rat SPR. The cells could also be immunostained with the antiserum and the staining was blocked by preabsorbing the antiserum with SPR393-407. Two approaches were used for immunocytochemistry in rat brain and spinal cord. In both cases the primary antiserum was diluted 1:20,000 and the avidin-biotin method of Hsu et al (Hsu et al., 1981) was used (See below). Some light microscopic immunocytochemical studies were performed on 15 μm cryostat sections of the brain (Figs. 1A,B). For this tissue TRITON-X 100 was used in all incubations to enhance penetration of the antisera into the tissue.

In the second protocol, seventy micron thick vibratome transverse (spinal cord and forebrain) or sagittal (spinal cord) sections were incubated in 50% ethanol in distilled water for 45 min, to improve antibody penetration (Llewellyn-Smith and Minson, 1992), washed in PBS and then blocked in 10% normal goat serum for one hour. Next the sections were

incubated in the SPR antiserum for 72 hour at RT. After extensive washing, the sections were incubated in a biotinylated secondary antibody for 2-4 hours and the avidin/biotin/peroxidase complex (Vectastain; Burlingame, CA) for one hour. To identify the immunoreaction product, the HRP was visualized with diaminobenzidine (DAB) and H_2O_2 with or without nickel intensification. The former results in a reddish-brown reaction product that is readily detected under dark field illumination. Controls for SPR immunostaining were run in parallel, using antiserum preabsorbed with 10^{-8} M SPR393-407. These sections contained no immunoreaction product (data not shown).

To localize the SPR immunoreactivity at the EM level selected immunoreacted vibratome sections through the cerebral cortex, striatum and spinal cord were osmicated (0.5%) for one hour, *en bloc* stained in 2.0% aqueous uranyl acetate for 30 min, dehydrated and flat embedded in Durcupan. Areas of interest were excised and mounted on resin stubs. Ultrathin sections were collected on Butvar coated nickel grids for observation in the electron microscope. On grids that contained sections through the superficial dorsal horn of the spinal cord we used a postembedding immunogold protocol to simultaneously localize the distribution of substance P- or GABA-immunoreactive terminals to study their synaptic relationship with SPR-immunoreactive profiles. Briefly, after washing in Tris-buffered saline, containing 0.1% TRITON-X 100, the sections were incubated overnight in either a rabbit anti-Substance P (1:2000) or anti-GABA antiserum (1:4000), both from Incstar (Stillwater, MN). After washing, the sections were incubated for one hour in a 15nm colloidal gold-labelled goat-antirabbit IgG (Amersham, Arlington Heights, IL). The sections were then stained with uranyl acetate and lead citrate and examined in the electron microscope. To determine the proportion of neuronal surface membrane that was immunoreactive for the SPR, we used a program from the Boulder Colorado High Voltage EM laboratory and measured the perimeter of labelled membrane, unlabelled membrane and percent of labelled membrane apposed by synaptic profiles.

RESULTS

General distribution of the substance P receptor- immunoreactivity

The distribution of SPR immunoreactivity was very similar to that reported in previous ^{125}I-SP-binding studies (Mantyh et al., 1989a; Quirion et al., 1983)(22, 26: Figs. 1A,B). A detailed description is in preparation. The receptor is located in some regions that have high concentrations of substance P, e.g., superficial laminae of the dorsal horn of the spinal cord (Fig. 2C) and the locus ceruleus and in areas that have minimal peptide, e.g., cerebral cortex (Figs. 1B-F) . Consistent with the radioligand binding studies, we detected no SPR receptor immunoreactivity in the substantia nigra, a region that contains the highest concentrations of SP in the brain. The very close correspondence of the receptor immunoreactivity and binding sites was also evident in the cerebellum, where we recorded bands of SPR immunoreactivity in the molecular layer of lobules 9 and 10, but not elsewhere.

Cellular distribution of the substance P receptor-immunoreactivity

Unappreciated in light level autoradiographic binding studies, but revealed in the present immunocytochemical analysis, is that the receptor is located on *all* parts of the cell body and dendritic tree; i.e., the receptor decorates the somatic and dendritic surface membrane of the neuron. In some cases, the labeling is of isolated cells, e.g., cerebral cortex (Figs. 1C-F) and spinal cord (Fig. 2C); in other cases, for example, the striatum (Fig. 1B) and the dentate gyrus of the hippocampus, we found a very dense meshwork of labeled cells and dendrites. Typically, the labeling was concentrated on the neuronal membrane, effectively outlining the neuron; some cytoplasmic labelling was also apparent. Although a similar pattern of labelling has been described in the striatum using a different antiserum (Shigemoto et al., 1993), the authors did not comment on the Golgi-like images that are produced.

Figures 1A and B compare the pattern of SP binding and SPR receptor immunoreactivity in the cerebral cortex and the striatum. Although areas of concentrated

binding in the cortex (arrowheads in Fig. 1A) can be recognized in the radioligand binding autoradiograms, the correspondence of these regions to the location of SPR-immunoreactive cell bodies could not be determined. Furthermore, although ^{125}I-SP binding can be detected throughout the depth of the cortex, it is clear that most of the binding is associated with dendritic arbors of a relatively small number of neurons (Fig. 1C). Immunochemically we determined that two SPR-immunoreactive cell types predominated in the cortex. In the region of laminae II and III, we found neurons that resembled the double bouquet and bitufted neurons (Jones, 1986), which have dorsoventrally arborizing dendritic trees (Figs. 1D,E) and are often GABA-immunoreactive (Henry et al., 1989). Some of the SPR-immunoreactive neurons had dorsally-directed dendrites that extended into and arborized within layer I. Many of the receptor-expressing neurons in the deepest part of the cortex, i.e. lamina VI, were fusiform, with dendrites that arborize horizontally (Fig. 1F).

The cellular labelling in the dorsal horn of the spinal cord, where the organization of SP-containing terminals and SP-responsive neurons has been well characterized (Henry 1976; Hokfelt et al., 1975), is particularly striking and informative. The most dense concentration of SP terminals, which derive largely from nociceptive, unmyelinated primary afferent fibers, is in the superficial dorsal horn, lamina I and the outer part of the substantia gelatinosa, lamina II, both of which contain nociresponsive neurons (Menétrey et al., 1977). We found that the largest concentration of SPR-immunoreactive neurons was in lamina I; the dendrites of these neurons arborized in lamina I. Although there is considerable overlap of SP and SPR immunoreactivity in lamina I, we found much less SPR in lamina II, the substantia gelatinosa. When present, it derived from dorsally-directed dendrites of neurons in lamina III. In some cases these dendrites extended into lamina I (Fig. 2C).

Subcellular distribution of the substance P receptor immunoreactivity

Electron microscopic analysis provided further detail concerning the surface labelling of neurons. Figure 2A illustrates an SPR-immunoreactive cortical neuron. Consistent with

a report on the striatum (Shigemoto, et al., 1993), we found that much of the neuronal surface is covered with immunoreaction product, indicating that the receptor is, in fact, widely distributed on the cell surface. Two properties of this labelling are, however, clearer at higher magnification (Fig. 2B). First, the surface labeling is not continuous. Second, most of the plasma membrane contained SPR that apposed unlabeled dendrites; a much smaller percentage apposed synaptic terminals, glial elements or other immunoreactive dendrites. Perimeter measurements established that 68% of the surface membrane of labelled cortical neurons (cell bodies and dendrites) was SPR-immunoreactive; only 9.0% of the SPR-laden membrane apposed a synaptic profile. These values were 72% and 3.7% for striatum and 65% and 15.5% for lamina I of the spinal cord.

Since this pattern of labeling is consistent with a peptide-peptide receptor mismatch at the synaptic level, we turned our attention to the dorsal horn, where a simultaneous analysis of the SPR and SP is more easily performed. Confirming the impression from light microscopy, we recorded some SPR-immunoreactive cytoplasmic labeling (Figs. 2D,E). As found in the cortex, however, the densest receptor labeling was found over the somatic and dendritic surface of the neurons, broken up by unlabeled surfaces of varying length. When a synapse contacted SPR-immunoreactive membrane, the dense DAB reaction product could be distinguished from the less electron dense postsynaptic density.

Electron microscopic double labeling established that some of the synaptic terminals in lamina I that were presynaptic to SPR-immunoreactive membrane were indeed SP-immunoreactive (Fig. 2D). The SP-immunoreactive terminals contained clear, round, and large dense core vesicles; only the latter were immunogold positive. In 90% of the SP-immunoreactive synaptic profiles, the dense core vesicles were located away from the synaptic junction, a finding consistent with previous results (De Biasi and Rustioni, 1988; Merighi et al., 1991) and with the report that dense core vesicles exocytose at sites distant from the active zone (Buma and Roubos, 1986; Zhu et al., 1986). Of 346 SP-immunoreactive terminals counted, 114 (33.3%) contacted a SPR-immunoreactive laden

profile, usually a dendrite. Finally, consistent with many other studies (Barber et al., 1978), we found that there is a dense GABAergic synaptic input in the superficial dorsal horn. Of particular interest is the observation that GABAergic synapses can contact membrane that is SPR-immunoreactive (Fig. 2E).

DISCUSSION

These studies demonstrate that there are significant difference between the cellular and subcellular distributions of classical transmitter receptors and the substance P receptor and provide important information that bears on the long-standing receptor-peptide mismatch problem. Previous studies that used ^{125}I-SP binding could not unequivocally identify the cellular location of the receptor. The combined light and electron microscopic analysis performed in the present study provides the cellular resolution necessary to establish that the substance P receptor is indeed neuronal; in the normal rat it is minimally if at all associated with glial or other non-neuronal elements (Mantyh et al., 1989b). We conclude that the mismatch that has been observed in radioligand binding studies reflects a real anatomical mismatch of the peptide neurotransmitter and the neuronal peptide receptor. It follows that peptide neurotransmitter and their receptors are, in fact, more long range in terms of their interactions than are classical neurotransmitter-containing synaptic terminals and their respective receptors, which usually maintain a tight association at a single postsynaptic target (Triller et al., 1985). Importantly, this property of the SPR is not limited to one CNS region. Rather intense and widespread SPR immunoreactivity in neurons was found throughout the brain and spinal cord. Our results differ somewhat from those of Moussaoui et al (Moussaoui et al., 1992). who used an antibody directed against the N-terminal of the SP receptor. Although they found intense immunoreactivity in the superficial dorsal horn, no cell body labelling was noted. They also reported axon and terminal, as well as dendritic, labelling. It will be important to determine whether the axonal labelling that they observed can be confirmed at the electron microscopic level.

Although the absolute amount of SPR, including the amount of surface membrane that bears receptor, varied in different neurons and in different brain regions, the percent of surface membrane in which the receptor was inserted was uniformally very high. The fact that nonsynaptic localization of the SPR was observed in structures as diverse as the spinal cord, cortex and striatum suggests that this property of the SPR is the rule, not the

exception. Importantly, the fact that most of the surface membrane of the neuron contains immunoreactive SPR, even when the apposing presynaptic element does *not* contain SP, suggests that the insertion of the receptor is not directed to particular regions of the neuronal surface. Indeed not only is the receptor located in surface membrane that is apposed by non-synaptic profiles (dendrites, etc.), but GABAergic terminals make contact with SPR-laden postsynaptic membrane. This arrangement differs from that observed in cerebellum, where the GABAergic receptor, although inserted in non-synaptic regions of granule cells, was *never* contacted by the presumed glutamatergic mossy fiber terminals (Somogyi et al., 1989).

As has been hypothesized for other peptide transmitter systems (Hamel and Beaudet, 1984) our results suggest that almost the entire surface of neurons that express the SP receptor may be acted upon by SP, presuming that SP can diffuse a considerable distance from its site of release. In fact, Duggan and colleagues (Duggan et al., 1990) demonstrated that primary afferent-derived peptides can diffuse several millimeters from their site of release in the substantia gelatinosa of the spinal cord. The diffusion of SP was dramatically enhanced by inhibitors of SP degrading-proteases (Duggan et al., 1992) or by calcitonin-gene related peptide which cooccurs with SP in primary afferent terminals (Merighi et al., 1991) and which also retards SP degradation (Le Greves et al., 1985). These findings suggest that the extent of diffusion, and thus the potential target neurons, are regulated under physiological conditions.

The fact that the dendritic architecture of individual neurons can be identified by the distribution of the receptor has also revealed important functional features of the neurons with which SP interacts. For example, neurons in lamina V of the spinal cord dorsal horn respond to noxious stimulation and receive direct SP contacts (De Koninck et al., 1992). By contrast, although neurons in laminae III and IV of the spinal cord typically do not respond to noxious stimulation (Menétrey et al., 1977), some express surface receptors that, if functional, can respond to SP (Fig. 2C), presumably derived from nociceptive primary

afferents that terminate dorsally, in lamina I and the substantia gelatinosa. This observation suggests that SP may modulate the firing of *non-nociceptive* neurons in lamina III. It is also apparent that although the SPR-immunoreactive cell bodies in the cortex are widely dispersed, their dendritic arbors cover large expanses of cortex. This fact, taken together with our observation that there are intimate appositions of SPR-laden dendrites, raises the possibility that widely dispersed SPR-containing neurons communicate through their dendritic arbors. Since there is very little SP in the cortex, the possibility must be considered that the peripheral terminals of trigeminal nerve primary afferent fibers that arborize around pial blood vessels overlying cortical layer I (Norregaard and Moskowitz, 1985) provide a source of peptide to the receptors on the dendrites of the SPR-laden neurons that extend to the surface of the cortex.

We, of course, cannot be certain that the receptor located distant from the SP containing synapse is functional; however, the fact that the pattern of ^{125}-I-SP binding and receptor located by immunocytochemistry are very similar indicates that the extrasynaptic receptor definitely binds ligand. This suggests that all sites of immunoreactive SP receptor are functional targets of released transmitter. It is of interest, in this regard, to address the significance of the *unlabelled* islands of membrane that are interspersed between the stretches of receptor-laden membrane. Conceivably, the receptor is never inserted into these regions. Alternatively, since there is considerable evidence that G-protein linked peptide receptors are internalized (Mazell et al., 1991; Yu et al., 1993), these islands may correspond to areas where recently released SP has bound to SPR, migrated to clathrin-coated pits and been endocytosed, leaving a "ghost" of recent synaptic activity behind. We are currently addressing this question using *in vivo* administration of SP in the presence and absence of SP antagonists. Assuming that ligand-receptor endocytosis is agonist-dependent, long term exposure of tissue to antagonist would conceivably result in a filling in of the islands with newly synthesized receptor.

In summary, these results demonstrate that the light microscopic mismatch that has been reported for peptide neurotransmitters and their receptors is evident at the synaptic level. Furthermore, the fact that the SPR almost completely decorates the somatic and dendritic surface of subpopulations of CNS neurons indicates that a significant proportion of the neuronal surface is a potential target of a peptide neurotransmitter. These results are significantly different from the transmitter receptor relationships that have been established for many other neurotransmitters, and reinforce the notion that cooccurring neurotransmitters in a synaptic terminal can target different postsynaptic elements.

Figure 1: A and B illustrate the very comparable pattern of SP receptor labelling in coronal sections of the rat cortex determined by autogradiographic localization of ^{125}I-SP binding sites (A) and immunocytochemistry using the SP receptor antibody (B). This is particularly clear for the striatum (st) and for the dorsolateral septum (lsd). In a few cases, clusters of silver grains (arrowheads, A) can be seen in the cerebral cortex. These probably correspond to cell bodies of SPR-containing neurons. The dark field photomicrograph (C) illustrates that there is a dense meshwork of SPR-immunoreactive dendrites that spans the depth of the cortex. These dendrites arise from a relatively small number of labelled neurons (arrows). The photomicrographs in D-F illustrate the Golgi-like staining that characterized SPR-immunoreactive neurons. D and E illustrate double bouquet-like neurons in layer III; F illustrates a fusiform cell in layer VI, just adjacent to the subcortical white matter and striatum (st). Calibration bars equal 1.0 mm in A and B, 65 μm in C and 100 μm in D-F.

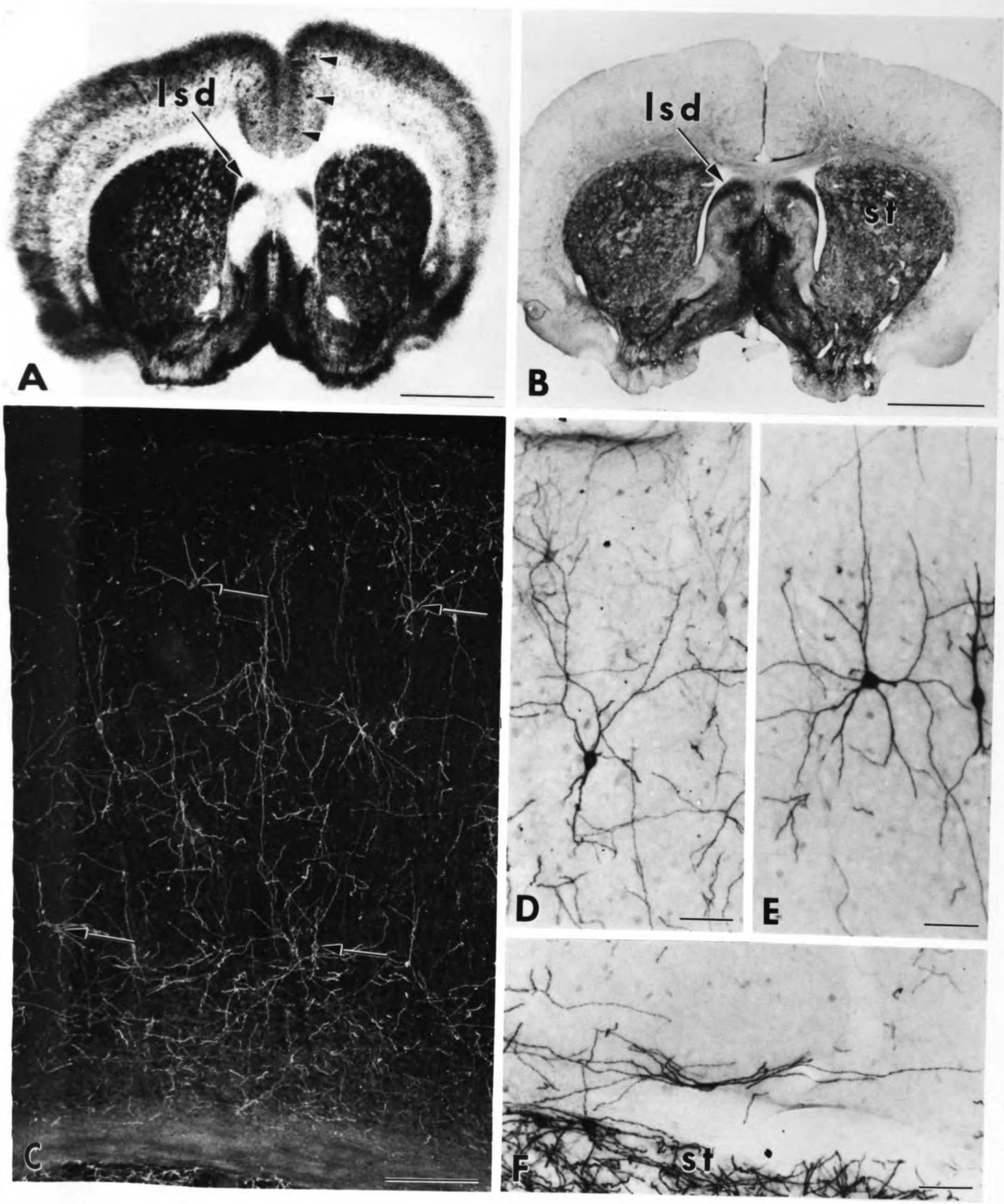
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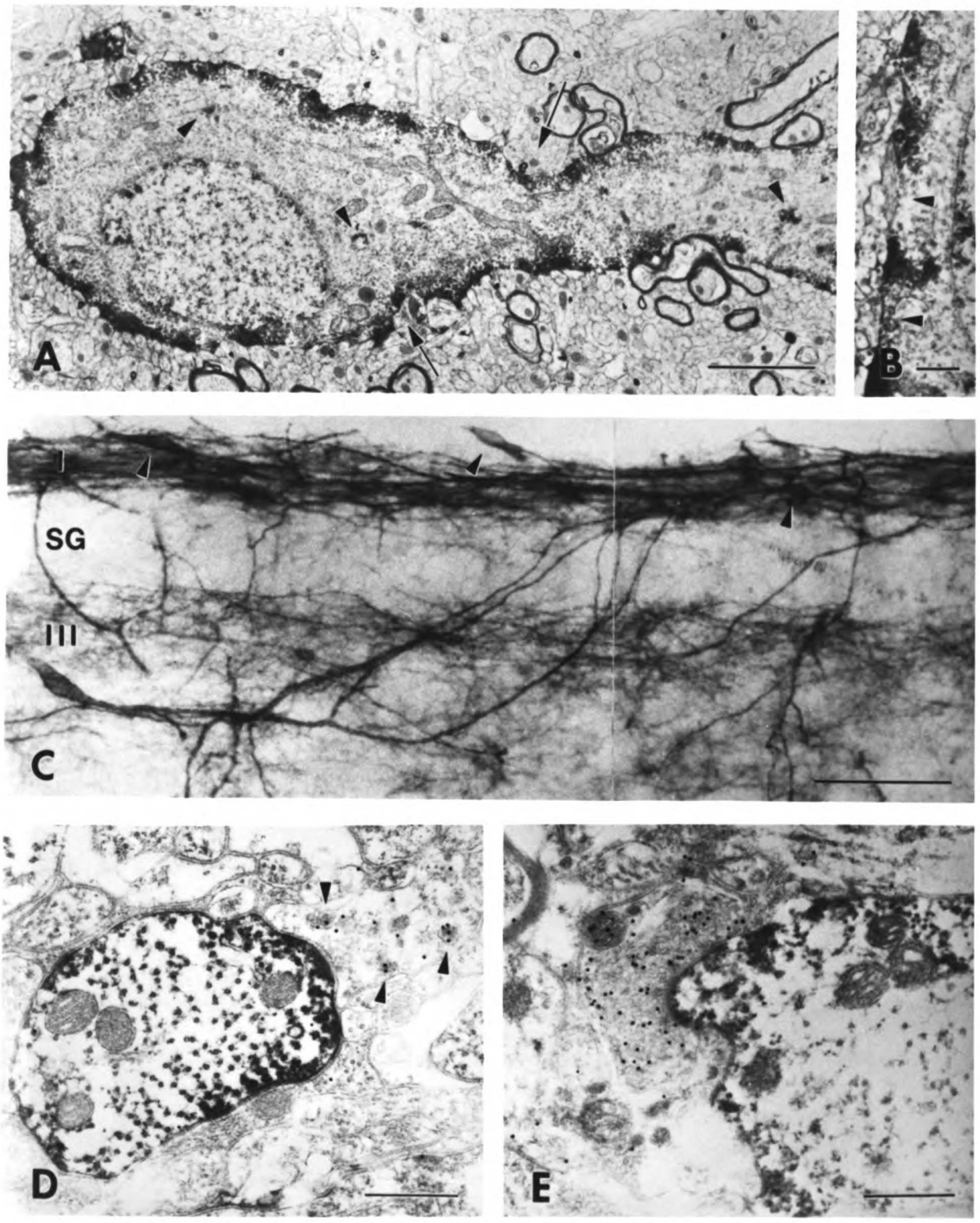
Figure 2: The electron micrograph in A illustrates a cortical SPR-immunoreactive neuron. Although the SPR immunoreactivity is concentrated on the neuronal surface, a few cytoplasmic organelles are also labelled (arrowheads). Despite the dense labelling, only two synaptic boutons contacted the cell body in this section (arrows). The inset in B illustrates the alternating patches of labelled and unlabelled (arrowheads) surface membrane. The sagittal section of the lumbar spinal cord (C) illustrates a densely labelled neuron in lamina III. The dendrites of this neuron extend through the substantia gelatinosa (SG) to lamina I, which contains numerous labelled cell bodies (arrowheads) and dendrites. The latter arborize in lamina I. The SG itself contains few labelled cell bodies. The electron micrographs (D,F), taken from lamina I of the dorsal horn, illustrate that *both* SP-immunoreactive terminals (D), identified by the colloidal gold labelling of dense core vesicles (arrowheads) and GABA-immunoreactive terminals (E), which contain round, clear vesicles, are presynaptic to SPR-immunoreactive dendrites. Calibration bars equal 4.0 μm in A, 1.0 μm in B, 200 μm in C and 1.0 μm in D and E.


A
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CHAPTER III

Morphological characterization of substance P receptor-immunoreactive neurons in the rat brain: Implications for nociception

Morphological characterization of substance P receptor-immunoreactive neurons in the rat brain: Implications for nociception

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Running Title: Substance P receptor in rat brain

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As described in the previous chapters, Substance P (SP), an eleven amino acid peptide, is an important signalling peptide implicated in several functionally distinct areas throughout the central nervous system (CNS). Comprehensive mapping studies in the rat brain, using either *in situ* hybridization to localize substance P mRNA or immunohistochemical localization of the SP peptide, demonstrated dense concentrations of SP throughout the brain and spinal cord; these studies have implicated SP in a variety of functional systems, including the processing of spinal cord motor output, olfaction, autonomic regulation, emotions and nociception (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978).

Substance P preferentially acts at the neurokinin-1 receptor (NK-1), a subtype within the tachykinin receptor family; SP binds the NK-1 receptor with a 100-fold higher affinity than related tachykinin peptides. Neurokinin A (NKA), which is usually cotranslated with SP from a preprotachykinin mRNA and is often co-localized with SP, acts preferentially at the neurokinin-2 receptor (NK-2); neurokinin B (NKB) has greatest affinity for the neurokinin-3 receptor (NK-3)(Helke et al., 1990). Since antagonists selective for the NK-1 receptor block the actions of substance P, we will refer to this receptor as the substance P receptor (SPR).

Despite the important information provided by the *in situ* hybridization and radioligand binding studies, neither of these techniques provides the resolution necessary to identify the populations of neurons that express the SP receptor. *In situ* hybridization labels cell bodies, but not dendrites, making it impossible to identify cellular morphology of neurons that express the receptor. Radioligand binding labels both cell bodies and dendrites, but the resolution does not reveal significant morphological features of individual neurons.

In two recent studies, we reported on the localization in the CNS of the SPR using an antibody directed against the carboxy terminal of the rat SP receptor. We found that the

SPR completely decorates the somatic and dendritic membranes of subpopulations of neurons throughout the rat spinal cord and brain (Liu et al., 1994). At the spinal cord level, the SPR was expressed on morphologically and topographically defined subpopulations of neurons (Brown et al., 1995). In the present study, we provide a detailed light microscopic analysis of the distribution of SPR-immunoreactive neurons throughout the rat brain.

METHODS

Characterization of the SPR antibody: The anti-SPR antibody was raised against a 15 amino peptide sequence (SPR393-407) in the C-terminal end of the rat substance P receptor (Vigna et al., 1994). The synthetic peptide sequence was conjugated to bovine thyroglobulin for immunization. The antiserum used in this study (#11884-5) recognized a protein band of 80-90kD on Western blots of membranes prepared from KNRK cells (Kristen murine sarcoma virus transformed rat kidney cells) transfected with the rat SPR. The transfected cells could also be immunostained with the antiserum; this staining was blocked by preabsorbing the antiserum with the SPR393-407 peptide. These observations taken together with the fact that our preliminary analysis revealed a high correspondence between the distribution of SPR immunoreactivity and ^{125}I-SP binding in the brain suggest that the antibody is selectively localizing the SP receptor. However, since we cannot rule out possible crossreactivity of the antiserum with other proteins, we refer to the staining as SPR immunoreactivity.

Immunocytochemistry: Experiments were performed on male Sprague-Dawley rats (260-300g; Bantin and Kingman, Fremont, CA). The rats were deeply anesthetized with ketamine (60mg/kg) and xylazine (6.0mg/kg) and then perfused intracardially with 50 ml of phosphate-buffered saline (PBS; 0.5 M) followed by 500 ml of 2% paraformaldehyde, 0.5% glutaraldehyde fixative in 0.1M phosphate buffer (PB). After perfusion, the brain and spinal cord were removed, postfixed for 5 hours in the same fixative and then cryoprotected in 30% sucrose in 0.1 M PB. Fifty micron thick sections, in either the coronal or sagittal

plane, were cut on a freezing microtome and then immunostained for the SPR using the avidin-biotin peroxidase method of Hsu et al. (Hsu et al., 1981) with nickel intensification of the chromogen, diaminobenzidine. The antiserum was routinely diluted to 1:20,000 and Triton X-100 was used in all incubation steps. Reacted sections were mounted on gelatin-coated slides and dried overnight. The following day, the sections were dehydrated and coverslipped with Cytoseal.

Preincubation of the primary antiserum with 10 μg/ml of $SPR_{393-407}$ abolished the staining. The distribution of immunoreactivity was analyzed according to the cytoarchitecture revealed in the rat brain atlases of Paxino and Watson (Paxinos and Watson, 1986) and Swanson (Swanson, 1992). Measurements of cell body diameters and dendritic length were taken directly from the microscope and given in approximate microns. When neurons were not round, the longest diameter was determined.

RESULTS

General observations on SPR immunoreactivity in the brain

The overall pattern of SPR immunoreactivity in the CNS is comparable to that observed in radiolabelled ligand binding studies. Figures 1A-J are low magnification photomicrographs that illustrate the wide distribution of substance P receptor immunoreactivity in the rat brain. We found the highest densities of SPR in several areas including: external plexiform layer of the olfactory bulb, striatum, lateral and medial septal nuclei, hippocampal dentate gyrus, subdivisions of the medial amygdaloid nuclei, parabrachial nucleus, locus coeruleus, dorsal raphe, nucleus of the solitary tract, and nucleus ambiguus. The highest concentration in the spinal cord was found in lamina I, particularly at sacral and lumbar segments, and around the central canal and in the intermediolateral cell column of thoracic segments (Brown, et al., 1995).

As previously described, the SPR is expressed over the entire somatic and dendritic surface membrane of labelled neurons. In the majority of cases, the density of SPR immunoreactivity in the neuronal membrane was sufficiently high so that the outline of the

soma and dendritic tree could be appreciated, which permitted morphological classes of SPR immunoreactive neuron to be characterized. In addition to morphological differences in different brain regions; however, we found significant differences in staining of individual neurons. For example, striatal neurons were consistently stained very darkly for the SPR; hippocampal CA1 neurons were very lightly labelled (Figure 5B and 7D).

In contrast to the "Golgi-like" SPR-immunoreactive staining pattern, several regions of the brain contained a much more diffuse pattern of SPR immunoreactivity, in which single neurons were difficult to identify or could not be be observed at all. In some cases this pattern consisted of darkly stained, presumably dendritic elements, but the overall neuronal morphology could not be identified. In other cases, the staining was like a diffuse cloud of immunoreactivity, with no discernible neuronal elements. Examples of this include a band of staining in the external plexiform layer of the olfactory bulb (Figures 1A, 2C) and subdivisions of the medial nucleus of the amygdala (Figure 8A). Occasionally, fine diameter processes could be made out in these patches of diffuse staining, which suggests that at least some areas of diffuse SPR immunoreactivity are actually dense conglomerates of lightly stained dendritic processes. Although this diffuse staining pattern is somewhat comparable to an axonal terminal field, we found no evidence for axonal SPR labelling. Although we observed axonal like varicose processes in several areas of the brain (Figures 3C, 4G, 5B) we could always trace these beaded processes back to a parent dendrite.

Olfactory Bulb

The densest concentration of SPR immunoreactivity in the main olfactory bulbs (MOB) was in the external plexiform layer (EPL) (Figure 1A). As described above, (Figure 2C) the dense SPR staining in the EPL had a diffuse appearance; neither cell bodies nor dendrites could be identified. Light, diffuse, SPR immunoreactivity was also observed outling the olfactory glomeruli. More posteriorly, we observed light labelling of the SPR in the mitral cell layer. A sparse population of neurons showed dense SPR-immunoreactive staining in the ependymal zone of the olfactory bulb. These neurons typically had small,

oval somata, ~5.0 μm diameter, and had a relatively simple dendritic tree, with few branches (Figure 2C). Many of the processes of the ependymal neurons were studded with bead-like swellings.

Associated Olfactory Areas

Anterior Olfactory Nucleus

The anterior olfactory nucleus (AON) contained a sparse population of darkly labelled neurons in each of its four subnuclei that surround the anterior commissure (ac) (Figure 1B, 2A,B). SPR-immunoreactive neurons in the medial, dorsal, lateral and posteroventral subnuclei of the AON were typically multipolar, with oval shaped cell bodies (~30 μm in diameter) from which at least four dendrites arose (Figure 2B). The dendritic processes were quite long, typically 300 μm in length, relatively unbranched, and were often varicose. These SPR-immunoreactive neurons were, for the most part, clustered subjacent to the plexiform layer that surrounds the anterior commissure. The dendrites of the AON neurons, although quite long, were also found predominantly in the cellular layer (figure 2A).

In the most caudal aspects of the AON, labelled neurons were still present in the posteroventral (PV) subnucleus, located ventral to the nucleus accumbens. Although these SPR-immunoreactive PV neurons appeared morphologically similar to those located in more rostral AON neurons, they were more lightly stained and more densely packed. Finally, in the transition zone from AON to piriform cortex, we recorded a small group of SPR-immunoreactive neurons. These neurons were most commonly bipolar with oval shaped somata and long dendritic processes that were both spiny and varicose. The dendrites were oriented dorsoventrally and extended over 400 μm in length.

Olfactory Tubercle

In the olfactory tubercle (OT), which includes the islands of Calleja (iC), we observed distinct populations of darkly stained SPR-immunoreactive neurons. The SPR neurons surrounding the iC were multipolar with somata of 40-50 μm diameter and short

dendrites of ~200 μm that extended ventrally, often to the surface of the brain. The majority of stained processes in the OT were studded with varicosities, throughout their length. The labelled neurons tended to cluster together, in a pattern that followed the the curved archictecture of the OT (Figure 3A). In general, the SPR-immunoreactive cell bodies were located in lamina II; their ventrally directed dendrites extended into lamina III of the OT.

Within the islands of Calleja, we found a dense population of small SPR-immunoreactive neurons that had oval-shaped somata (~15 μm in diameter) and dendrites that were clustered within the islands (Figure 3A). The majority of these dendritic processes were heavily varicose. Staining in the OT and especially in the islands of Calleja was present throughout their rostrocaudal extent; At the level of the septal nuclei, the main nucleus of the islands of Calleja were particularly densely stained.

Piriform cortex and endopiriform nucleus (EPv)

The piriform cortex was largely devoid of SPR immunoreactivity. Occasionally, SPR-immunoreactive neurons similar to those observed in the neocortex were identified in the deeper laminae of the piriform cortex; this staining pattern continued into the endopiriform nucleus, which itself contained a moderate amount of labelling, particularly in its ventral part. In the EPv nucleus staining was predominantly found on a complex of varicose dendrites.

Cerebral Cortex

The cerebral cortex contained a small, but very distinct population of SPR-immunoreactive neurons. The expansive and heavily labelled dendritic trees of most SPR-immunoreactive neurons in the cortex simplified their morphological characterization; two types of labelled neurons predominated. Lamina VI and the ventral most portion of lamina V contained the greatest density of SPR-immunoreactive neurons and processes. Lamina VI contained a distinct population of heavily labelled bipolar neurons; the cell bodies were fusiform, ~ 20 μm in diameter, with dendrites oriented parallel to the white matter; the dendrites could be followed up to 600 μm in the transverse plane (Figure 4F). Many of the

labelled neurons in the deep laminae were highly varicose (Figure 4G). In the more superficial portion of lamina VI and in the deeper portion of lamina V, we observed a population of SPR-immunoreactive neurons that resembled the double bouquet neurons described in Golgi preparations. These neurons had round somata that measured 20-30 μm and long, relatively unbranched dendrites that were directed dorsoventrally, up to 400 μm in length; the dendrites were commonly spiny and varicose. This population of SPR-immunoreactive neurons was most commonly observed in medial cortical areas, adjacent to the cingulate bundle (Figure 4D). Only rarely did we observe SPR-immunoreactive pyramidal neurons (Figure 4C). Interestingly, using a different antiserum directed against the SPR, Nakanishi and colleagues found that many SPR-immunoreactive double bouquet neurons contain GABA, which suggests that these are an inhibitory subpopulation of cortical neurons (Kaneko et al., 1994).

A second population of SPR-immunoreactive neurons was scattered within more superficial laminae, II and III. Some of these neurons resembled the double bouquet neurons that we noted in lamina VI (Figure 4B). Other SPR-immunoreactive neurons in lamina II-III were more multipolar, with dendrites that radiated in all directions from the cell body; the dendrites, which were often spiny and varicose, measured up to 500 μm in length. SPR-immunoreactive neurons in the more superficial laminae of the cortex were more lightly labelled than were neurons in deeper laminae (Figure 4A). Finally, we noted a light, diffuse labelling of the white matter of the corpus callosum. We also recorded lightly labelled cells scattered even throughout the callosum. We presume that these are astroglial cells (see below, cerebellum).

Nucleus Accumbens

The nucleus accumbens (Acc) contained a dense network of immunoreactivity, consisting mainly of large, heavily labelled dendrites and scattered neurons (Figure 3B). The neurons were typically multipolar with fusiform cell bodies that measured ~ 40 μm in the longest diameter; the dendrites were relatively short, ~ 200 μm, however, the complex

network of dendrites made it difficult to measure accurately (Figure 3C). Most of the labelled processes were beaded.

Caudate-Putamen

As described previously, the caudate-putamen (CPu), like the nucleus accumbens, also contains a dense meshwork of densely stained neurons. Figure 5A illustrates the pattern of SPR immunoreactivity in the CPu. The ventral CPu was more uniformly filled with labelled neurons. Although the dorsal CPu had small areas devoid of labelled neurons, we could not discern whether this corresponded to the characteristic patch-matrix organization of the striaturm. We distinguished two populations of SPR-immunoreactive neurons (Figure 5B). Some CPu neurons were large and multipolar with fusiform cell bodies, ~40 μm in diameter. The density of neurons made it difficult to assess the lengths of dendrites. Most appeared to be approximately 200 μm in diameter. The second group of CPu neurons was smaller, with round cell bodies, between 20 and 25 μm in diameter. The majority of the CPu dendrites were varicose.

Globus Pallidus

Although the lateral segment of the globus pallidus (GP) contained considerable SPR immunoreactivity, we did not observe labelled cell bodies (Figure 5C). Rather, the SPR immunoreactivity in the external GP consisted of a diffuse patch of staining comparable to that observed in the external plexiform layer of the olfactory bulb (figure 5D). By contrast, the internal segment (Figure 5C) of the GP contained a dense population of darkly labelled neurons that were morphologically similar to the SPR-immunoreactive neurons in the CPu.

Septum

Several areas throughout the septum exhibited a high density of SPR-immunoreactive neurons. In the anterior septum, we observed SPR immunoreactivity in the medial septal nucleus (MS) and in the dorsal (LSd) and intermediate (LSi) parts of the lateral septal nucleus (Figure 6A). The MS contained scattered, round cell bodies, approximately

15 μm in diameter, and a dense mesh of varicose dendritic processes (Figure 6C). In the medial aspects of the LSi, surrounding the MS, we recorded additional clusters of round cell bodies, ~20μm in diameter. A plexus of heavily labelled, often varicose, dendrites radiated from this medial group of neurons, into the more lateral aspects of the LSi. The dorsal aspect of the lateral septum contained a small population of medium sized, round, multipolar neurons (20-30 μm in diameter). These were lightly labelled giving the region a relatively diffuse appearance Figure 6B). The dendrites of these neurons extended up to ~200 μm in the transverse plane and were often beaded. At the level where the nucleus of the diagonal band merges with the MS, the lateral septum was largely devoid of staining; the only exception were two prominent patches of SPR-immunoreactive neurons in the LSi. Within the caudalmost part the LSi, surrounding the precommissural fornix, we recorded a large patch of SPR-immunoreactive neurons (Figure 6A).

A different pattern of SPR immunoreactivity was noted in the caudal septum. At the level of the triangular septal nucleus (TRS), the central portion of the TRS was devoid of immunoreactivity, but the periphery contained a dense complex of labeled processes (figure 1D). The dendrites were somewhat spiny and extended on average 300 μm in a dorsolateral direction from the TRS. A few cell bodies (20-30 μm) were observed in this region. In the midline, dorsal to the TRS, we recorded a very small group of large, multipolar neurons, densely labeled for the SPR. These neurons had round cell bodies (~ 50 μm diameter) and fine processes, some of which were varicose.

Nuclei of the basal forebrain

Substantia Inominata

The rostral portion of the substantia inominata (SI) contained a densely packed, heterogenous population of SPR-immunoreactive neurons that made it easily distinguishable from the adjacent nucleus accumbens and olfactory tubercle (Figure 1C). One group of labelled neurons in the SI were multipolar with 20 μm diameter, fusiform cell bodies; dendrites of these neurons could be as long as 300 μm and were often studded with

varicosities and spines. A second population of SPR-immunoreactive SI neurons had much smaller, round cell bodies (~ 10 μm diameter) that emitted rather short, local dendritic arbors.

In the most caudal aspects of the SI, the density of SPR labeled neurons was greatly reduced. In fact, at the point where the septum disappears and the hippocampus emerges, the SI is outlined by SPR-immunoreactive neurons. At this level, the labelled neurons have fusiform or oval shaped somata and varicose processes; these neurons are more lightly stained than those located rostrally, in the SI, itself.

Nucleus of the diagonal band

The nucleus of the diagonal band (NDB) was also filled with a dense mesh of SPR-immunoreactive cell bodies and processes. The cell bodies were small, ~ 20 μm, and the dendrites were thin, relatively short (200 μm) and studded with varicosities (Figure 6D). Neurons in the NDB were more lightly stained for the SPR than were the labelled neurons in the medial septum.

At caudal levels, as the NDB disappears and the magnocellular preoptic nucleus (MA) emerges, we recorded a population of darkly stained neurons in the MA. Labeled cells in the MA have 15-20 μm oval shaped somata; the dendrites were short, less than 200 μm, thin and were highly varicose.

Bed nucleus of the stria terminalis

In light of previous binding studies that found significant receptor labelling throughout the bed nucleus of the stria terminalis (BST) we were surprised to find this area largely devoid of SPR immunoreactivity (Figure 1D). The exception to this pattern was in the principle nucleus of the BST, adjacent to the anterior commissure, where we recorded scattered, lightly labelled neurons. We also recorded a complex of short, fine, intensely labelled processes surrounding the anterior commissure and fornix, but these were not associated with any of the BST subnuclei.

Hippocampus

We observed SPR immunoreactivity in all subregions of the hippocampal formation (Figure 7A). The highest density of SPR immunoreactivity was in the granular layer of the dentate gyrus (DG). The labelled cells in the dentate gyrus had large fusiform cell bodies (~40 μm) with thick spiny dendrites (Figure 7C). Dorsal to the DG, we recorded a small population of presumed basket neurons that were very lightly labeled for the SPR (Figure 7C). These neurons had a round cell body (30 μm diameter) and dorsally directed primary dendrites (Figure 7B).

In the CA1 field of the hippocampus, we observed a dense population of labeled cell bodies in the stratum lacunosum-moleculare, along the border of the hilar fissure (Figure 7A). These neurons were moderately stained and had round cell bodies (~ 20 μm) long, apical dendrites that extended dorsally, more than 400 micron, reaching beyond the pyramidal layer.

Within the vicinity of the pyramidal cells of CA1, we also observed a small population of multipolar neurons, with large fusiform cell bodies (40 μm) and long apical and basal dendrites that extended into the stratum radiatum and stratum oriens; these neurons were typically much more lightly stained (Figure 7B). In the fasciola cinerea (FC; Figure 7D), the most medial portion of CA1, darkly stained neurons of similar morphology but small somata size, 30 μm in diameter, were clustered around the pyramidal cell layer. The labeled dendrites in both CA1 and FC were often varicose. The CA2 contained SPR immunoreactive pyramidal neurons, but they were very lightly stained. Finally, the CA3 field contained a complex network of SPR immunoreactive neurons. At more caudal levels these neurons were ordered subjacent to the pyramidal layer, in the stratum lucidum of CA3. The neurons were of medium size (25 μm cell bodies) and were moderately immunoreactive for SPR; the dendritic processes were thick, somewhat spiny, and extended from 300-400 μm.

Amygdala

Several amygdaloid subnuclei located around the medial border of the amygdaloid complex, including the cortical, medial, and posterior nuclei expressed intense SPR staining. In contrast, many of the remaining amygdaloid nuclei were identified by the absence of SPR staining.

Cortical nucleus

We recorded very dense SPR immunoreactivity in the anterior portion of the cortical nuclei of the amygdala (COA; Figure 8B). Its dorsal aspect contained a moderately dense population of immunoreactive round cell bodies, ~ 35 μm in diameter. The dendrites of these neurons, for the most part, extended from 300-400 μm ventrally, into the ventral portion of the COA; these processes were thin and highly varicose (Figure 8C).

Medial nucleus

The medial nucleus was the most densely stained for the SPR in the amygdala. In the posterioventral portion of the medial amygdala (MEApv), we observed a dense complex of SPR immunoreactivity. For the most part the staining consisted of a very dense patch of diffuse SPR immunoreactivity (Figure 8A). The posteriodorsal portion of the medial amygdala (MEApd) contained a dense meshwork of SPR-immunoreactive fibers and some cell bodies (Figure 8D). In this case, the staining decorated small round cell bodies and complex arrays of dendrites.

Basomedial nucleus

A sparse population of fusiform neurons were moderately labeled in rostral levels of the basomedial nucleus (BMA). The cell bodies of these neurons measured 40 μm in diameter and the dendrites of these neurons were highly varicose.

Basolateral, lateral, and central nuclei

In contrast to the dense staining of cortical and medial nuclei, the basolateral (BLA), lateral (LA), and central (CEA) nuclei of the amygdala were generally devoid of SPR

immunoreactivity, except for the appearance of a few SPR-immunoreactive processes located at the borders of these subnuclei, effectively outlining them (Figure 8D). Most of the staining in these border regions was expressed on a network of beaded processes; some oval shaped cell bodies, ~ 30-40 μm, were also present in the areas surrounding these subnuclei of the amygdala. One exception to this paucity of staining was the presence of a diffuse band of SPR immunoreactivity in the central nucleus; this staining, however, did not conform to a specific subnucleus within the CEA.

Posterior nucleus

We also observed a dense band of SPR immunoreativity in the posterior nucleus of the amygdala (PA). The quality of the staining was almost identical to that observed in the MEApv.

Hypothalamus

For the most part, we could not match the pattern of SPR immunoreactivity in the hypothalamus with the traditionally identified cytoarchitectural regions. Rather, the hypothalamic staining typically was located in regions that bounded the hypothalamic subnuclei, effectively outlining them.

At the preoptic level of the hypothalamus, SPR staining was localized primarily around the third ventricle. A small cluster of SPR neurons appeared dorsal to the third ventricle in the area of the median preoptic nucleus (MEPO); these neurons had very small (~ 10 μm), round cell bodies and short fine dendritic processes, that were occasionally varicose. We identified another population of SPR-immunoreactive neurons along the ventral portion of the third ventricle, in the region of the suprachiasmatic nucleus. These neurons were morphologically similar to those found in the MEPO. A small cluster of tiny SPR-immunoreactive neurons were lightly labeled in the medial preoptic nucleus (MPO); these had 10 μm diameter cell bodies and short, fine processes.

The suprachiasmatic nucleus (SCH) and the anterior hypothalamic nucleus were noticeably devoid of SPR immunoreactivity (Figure 9A). However, the area surrounding

the SCH and the AHNa, which partially includes the anterior hypothalamic area, contained clusters of SPR-immunoreactive neurons with small round cell bodies (10 μm) and fine dendritic processes, 100-200 μm in length.

The paraventricular nucleus (PVH) was also devoid of SPR immunoreactivity (Figure 9B). However, in areas adjacent to the PVH, that include the anterior hypothalamic nucleus, central part (AHNc) and subparaventricular zone (SBPV), there is a population of lightly labeled neurons. The labeled neurons in the AHNc and SBPV had round, 25 μm cell bodies and fine varicose processes, about 200 μm in length. Within the dorsal AHNc, we noted a small crescent shaped area that was filled with small, intensely labelled SPR-immunoreactive neurons. At these same levels, we recorded a small number of intensely SPR-immunoreactive neurons in the median eminence; these neurons had large cell bodies (~ 50 μm in diameter) with thick, spiny, bipolar dendrites that extended laterally ~ 200 μm.

The lateral hypothalamic area (LHA) was marked with moderately labeled multipolar neurons that had oval cell bodies (10-15 μm) and varicose processes that measured 200 μm in length. The ventromedial hypothalamus was devoid of SPR immunoreactivity. Dorsal to the dorsomedial hypothalamus, a region that we could not identify in cytoarchitectural atlases, we noted a dense network of lightly labeled processes; occasionally 20 μm oval cell bodies were observed. This region appeared to fuse laterally with the zona incerta , but because it was present at the midline, we could not unequivocally identify it. At these levels, the median eminence still contained a few large neurons that densely expressed the SPR.

Two areas associated with the mammillary bodies contained SPR immunostaining. In the supramammillary nucleus (SuM), we recorded a small population of neurons with oval shaped cell bodies (~10 μm) and fine dendritic processes that were lightly stained for the SPR. The lateral portion of the medial mammillary nucleus (ML), contained a diffuse band of SPR immunoreactivity. These two areas of SPR immunoreactivity formed encircled the mammillary peduncle.

Thalamus

Although the majority of thalamic nuclei did not express SPR at appreciable densities, there were a few notable exceptions. Thalamic nuclei in the ventral and medial subdivisions were moderately stained for the SPR.

Anterior thalamic nuclei:

In the most anterior thalamic areas, the predominant region of SPR immunoreactivity was observed in a thin lamina that separates the nuclei of the anterior thalamus from the reticular thalamic nucleus (RT). The staining in this region consisted of a complex of thin processes that coursed parallel to the boundary between the anterior thalamus and the RT. Some small lightly stained cell bodies were observed within this complex of SPR-immunoreactive processes.

Medial thalamic nuclei:

The medial and lateral habenula (Hb) were moderately stained with a fairly dense population of SPR-immunoreactive neurons that were small ,with oval cell bodies (20 μm) and dendritic processes that were highly varicose. Ventral to the habenula, near its most anterior limits, and dorsal to the third ventricle, we observed bilateral patches of SPR immunoreactivity in the region of the central medial nucleus (CM; Figure 10A). Neurons in this area were lightly labeled and had ~30 μm oval cell bodies and thick dendritic processes. At the same level, we noted a small population of densely stained neurons along the lateral edge of the paraventricular nucleus of the thalamus (PVT; Figure 10A). The SPR-immunoreactive neurons that surrounded the PVT had 20 μm oval cell bodies and thick processes, some of which were varicose.

At the most caudal level of the habenula, and just ventral to it, we observed a diffuse network of SPR-immunoreactive processes along the midline, ventral to the Hb. These cells surrounded the fasciculus retroflexus in the area of the parafascicular thalamic nucleus (Pf; Figure 10 A,B) and the intermediodorsal thalamic nucleus (IMD; Figure 10B). Most of these cells were lightly labeled. Within this network of labelled processes, we occasionally

observed labeled cell bodies, ~15 μm diameter. In the subparafascicular nucleus, located immediately ventral to the fasciculus retroflexus, and in more ventral areas around the third ventricle, we recorded an increased density of darkly stained neurons. The latter had had large fusiform cell bodies (~ 40 μm) and long, up to 400 μm, dendritic processes that were oriented mediolaterally. Finally, we observed a small, but distinct patch of diffuse SPR immunoreactivity in the central lateral nucleus (CL).

Ventral thalamic nuclei:

The ventrobasal complex of the thalamus was largely devoid of immunoreactivity for the SPR. We identified a very light and diffuse SPR staining in the medial and lateral aspects of the ventral posterior thalamic nuclei and posterior complex, but we could not discern labelled cell bodies.

The lateral geniculate

The dorsolateral geniculate was largely devoid of immunostaining; however, we recorded very dense immunostaining in the ventrolateral geniculate (VLG; Figure 10C). The parvocellular region of the VGN was filled with densely immunoreactive processes; large cell bodies, measuring 40 μm in diameter, were occasionally observed. The magnocellular region of the VGN contained a small population of darkly labeled neurons; these neurons had pyramidal-shaped cell bodies and dendritic processes that extended 100-200 μm in length (Figure 10D).

Posterior Thalamic Nuclei

Several of the posterior thalamic nuclei and related nuclei that surround the medial geniculate (MG), which itself was devoid of immunostaining, contained light to moderate SPR immunostaining (Figure 1G-H.) Specifically, the posterior intralaminar nucleus (PIL) and the peripeduncular nucleus (PP) contained thin processes that were lightly labeled. We also observed a thin band of staining medial to the medial geniculate, in a region that corresponds to the posterior limitans nucleus PLi, which separates the MG from more medial structures. Within the PLi, we recorded a complex of fine fibers that were lightly

stained for the SPR.

Brainstem regions:

Superior and inferior colliculi

Both the inferior and superior colliculi (Figures 1H,I) contained moderate concentrations of SPR immunoreactivity. In the inferior colliculus, we recorded numerous light patches of SPR immunoreactivity that contained a heterogenous group of lightly stained neurons. These neurons were medium to large in size with very fine dendritic processes.

In the superficial gray layer of the superior colliculus (SC), we observed a diffuse band SPR immunoreactivity. Although individual cell bodies were undetected, fine immunoreactive dendritic processes could be discerned. The internal gray layers contained a population of small neurons with round cell bodies and thin dendritic processes that were moderately labelled. The densent staining was in the most superficial sublayer.

Interpeduncular Nucleus

The interpeduncular nucleus (IPN) was largely devoid of staining. Except for a few fine labeled dendrites in the caudal IPN, this region contained no SPR immunoreactivity.

Ventral and dorsal tegmental nuclei:

The ventral tegmental nucleus (VTg) contained a small group of neurons with round bodies, ~20μm in diameter, that were moderately immunoreactive for SPR. The posterior regions of the dorsal tegmental nucleus (DTg) were moderately labeled. The staining in this nuclei was diffuse; we could not identify individual neurons.

Periaqueductal gray

The periaqueductal gray (PAG) was distinguished by its having a background distribution of diffuse SPR immunoreactivity throughout. Within this background staining, we observed discrete populations of SPR-immunoreactive neurons in the ventrolateral and dorsal portions of the PAG (Figure 11A). Most of the neurons were bipolar, with round

cell bodies (20 μm) and dendrites that extended local arbors. Figure 11B illustrates the dense concentration of SPR immunoreactivity in the dorsal PAG, just ventral to the posterior commissure; this region contained a dense bundle of darkly stained processes.

Nucleus cuneiformis

The nucleus cuneiformis (NC), at the level of the superior colliculus contained a dense meshwork of SPR-immunoreactive processes (Figures 1I, 14A). These densely labeled processes were thin and occasionally beaded. Small, darkly stained cell bodies were also found within theis region. Although anatomical studies have often recognized a continuity of staining patterns of the ventrolateral PAG and NC, there was a clear separation in the distribution of SPR-immunoreactive neurons in these two regions.

Raphe nuclei

We recorded significant levels of SPR immunoreactivity in most of the raphe nuclei of the brainstem; the density of the label, however, differed considerably in different raphe nuclei. The dorsal raphe nucleus (DR) located in the ventral portion of the central gray was the most densely labelled for the SPR (Figure 1I, 11C). We identified small darkly stained neurons (less than 10 μm in diameter) on the midline of the DR and dense bundles of very fine, but darkly stained processes that extended ventrally from the nucleus. In the raphe pontis, we recorded only modest amounts of SPR staining, in a small population of neurons. The labeled neurons had small cell bodies with fine dendritic processes. The nucleus raphe magnus in the rostral medulla contained a small, but very extensively labelled population of SPR-immunoreactive neurons (Figure 14B). These neurons had large fusiform cell bodies (40-50 microns) with dendrites that arborized mediolaterally for 200-300 μm (Figure 14C). The raphe obscurus contained a few large, multipolar neurons that were moderately stained for the SPR. The dendrites of these neurons arborized ventrally for ~ 200 μm. Finally we found no SPR-immunoreactive cell bodies or processes in the raphe pallidus, throughout its rostral caudal extent.

Locus Coeruleus and Parabrachial Nuclei

The locus coeruleus contained the densest SPR immunoreactivity in the central nervous system. The immunolabelling was concentrated over small, very heavily labelled oval cell bodies, generally less than 10 μm in diameter. The density of labelling was so great that the individual dendrites could not be appreciated in transverse sections through the LC (Figures 1J, 12C). Sagittal sections, however, revealed many fine densely stained processes running in a rostrocaudal direction (Figure 12D). The density of labelling dropped off significantly in the subcoeruleus. located immediately ventral to the LC (Figure 12A). Finally, we observed intense SPR immunoreactivity in both the lateral and medial parabrachial (PB) nuclei. The cells that expressed the SPR were morphologically similar to those in the locus coeruleus. They had small cell bodies with fine processes that coursed rostrocaudally (Figure 12B).

The A5 cell group

The A5 cell group (many neurons of which are noradrenaline containing) contained a dense cluster of SPR-immunoreactive neurons (Figures 1L; 14D). The SPR staining was concentrated in intensely labeled multipolar neurons that had rather small cell bodies, ~10μm in diameter, and thin, but heavily labelled dendritic processes that arborized throughout the A5 region, often extending dorsally and ventrally beyond the confines of the nucleus.

Reticular Nuclei

The caudal pontine reticular nucleus contained a scattered population of very large SPR-immunoreactive neurons; most were concentrated in the ventrolateral portion of the nucleus. These large neurons were multipolar and had relatively short dendrites; the density of staining was moderate (Figure 1J). The dorsal paragigantocellularis nucleus, located just lateral to the medial longitudinal fasciculus at the level of the rostral medulla, contained a small population of large SPR-immunoreactive neurons, cell bodies ~ 40 μm in diameter. Many of these neurons had long dendrites that arborized ventrally, reaching up to 400 μm. Although we found little staining in the nucleus gigantocellularis at the level of the rostral

medulla, we recorded significant numbers of SPR-immunoreactive neurons in the gigantocellular nucleus, pars alpha, which is located just dorsal to the pyramids and adjacent to the nucleus raphe magnus. The morphology of the neurons was comparable to that of the SPR-immunoreactive neurons in the NRM; the cells were large, fusiform and had mediolaterally oriented dendritic arbors (Figures 14B,C).

The parvocellular reticular nucleus (PCRt), located medial to the spinal trigeminal nucleus, contained a loose network of smooth dendritic processes. This region also contained a few small, round SPR-immunoreactive cell bodies (less than 10 μm) with dendrites that arborized in all directions. In the rostroventrolateral reticular nucleus, an area that includes the C1 group of epinephrine-containing cells, we observed a small group of oval shaped cell bodies (~10 -15 μm in diameter) and a network of relatively small diameter dendritic processes.

The dorsal medullary reticular nucleus, located ventral to the largely unlabelled dorsal column nuclei and medial to the nucleus caudalis of the spinal trigeminal nucleus, also contained a network of moderately labelled dendrites that were of fine diameter and occasionally beaded. This region contained scattered, small, round SPR-immunoreactive cell bodies and a small group of large fusiform, immunolabelled neurons. The dendritic processes of the later neurons were relatively long, arborizing in a dorsomedial direction.

Inferior Olive

In the dorsal and medial and accessory inferior olivary nuclei, we observed a moderate density of SPR immunoreactivity. The medial accessory nuclei contained a very diffuse pattern of SPR immunoreactivity within which we could not discern labelled cell bodies.

Cerebellum

With the exception of lobules 9 and 10 of the cerebellum, we found no staining in the cerebellum cortex. In transverse section, the SPR immunoreactivity in lobules 9 and 10 formed very dense stripes that spanned the molecular layer; these were separated by areas

devoid of staining (Figures 1K, 13). This pattern is reminiscient of the zebrin stripes. In sagittal sections we noted that the labelling was continuous through the folium. We could not identify the structres that were labelled within each stripe; no cell bodies were observed. The cerebellar white matter was also uniformly stained and contained large concentrations of lightly labelled, presumed astrocyte cell bodies (Figure 13). This region and the corpus callosum (see above) were the only white matter regions that was contained SPR immunoreactivity, which suggests that it is not nonspecific background staining.

Nucleus of the solitary tract and the dorsal motor nucleus (DMV)

The nucleus of the solitary tract (NTS) contained very dense SPR immunoreactivity. This was particularly true of the medial nucleus at levels rostral to the obex (Figures 1M, 14E). This area contained 20-30 μm oval cell bodies and 200-300 μm dendritic processes that typically arborized in the mediolateral plane. Less densely packed neurons of similar morphology were found in the ventrolateral subdivision of the NTS, particularly at more caudal levels. There was very low levels of staining in the commissural nucleus; the area postrema was devoid of staining.

Ventral to the NTS, we recorded moderate SPR immunoreactivity in tightly packed neurons of the DMV. This region also contained fine, lightly labelled dendritic processes that appeared to be oriented in the rostrocaudal plane.

Nucleus ambiguus

The nucleus ambiguus contained a high concentration of heavily labelled neurons (Figure 1M). The density of staining made identification of the majority of neurons difficult (Figure 14F), however, we could discern small round cell bodies (~10 μm) near the borders of the nucleus. Labelled processes were thin and, based on analysis in sagittal section, we determined that they ran primarily in the rostrocaudal direction.

Trigeminal Complex

The subnucleus caudalis of the spinal trigeminal nucleus expressed SPR immunoreactivity in a pattern similar to that observed in the spinal cord. SPR staining was

concentrated in neurons of the marginal zone, lamina I; the majority of the labelled cells had fusiform cell bodies (up to 40 μm in diameter) and dendrites that arborized around the curvature of the marginal layer. The substantia gelatinosa, lamina II, was noticeably devoid of SPR-immunoreactive neurons. The magnocellular layer, which corresponds to laminae III and IV of the spinal cord dorsal horn, contained a small population of large SPR-immunoreactive neurons; these neurons were typically multipolar or pyramidal-shaped, with long dorsally directed dendrites that occasionally reached lamina I. The subnucleus oralis and interpolaris showed little SPR staining.

DISCUSSION

General observations

In the present study we demonstrate the substance P receptor is expressed by subpopulations of neurons throughout the CNS. Importantly, we found a high correspondence between the pattern of SPR immunoreactivity and the distributions of SP binding sites reported in previous studies; this provides strong evidence that the antibody is selective for the substance P receptor. A great advantage of the present immunocytochemical analysis is that because the SPR is expressed throughout the somatic and dendritic surface membrane, we were able to identify morphologically distinct subpopulations of SPR-immunoreactive neurons within regions of the CNS that contain heterogeneous populations of neurons. For example, although previous studies revealed a dense concentration of SP binding in the striatum, we found that most of the labelling is associated with the dendritic arbors of a relatively small percentage of the neurons in this region. In other cases, we established that areas where binding was of low to moderate density, contained a distinct population of well labelled SPR-immunoreactive neurons; this was true for several subnuclei of the brainstem reticular formation and the nucleus raphe magnus and was true for most regions of the cerebral cortex. The fact that there is significant dendritic labelling emphasizes the additional information that immunocytochemical characterization provides over studies that mapped the receptor using *in situ* hybridization. The latter studies only define the distribution of *cell bodies* that express the receptor.

In addition to revealing a good correspondence between areas of high binding and areas of dense SPR immunoreactivity, we confirmed the reported areas of mismatch between the distribution of SP and the NK-1 receptor (Shults et al., 1984), notably the substantia nigra. On the other hand, we found another area of significant mismatch not revealed in binding studies, namely the interpeduncular nucleus of the midbrain, which receives a dense SP input from the habenula, but which contained only a few SPR-immunoreactive neurons. Taken together with our demonstration that the receptor is

expressed on large regions of plasma membrane that are not apposed by synaptic processes, these results provide further support for the hypothesis that peptide neurotransmitters can diffuse from their site of release to act at "nonsynaptic" receptors (See below).

Absence of SPR-immunoreactive axons

We found no evidence for axonal labelling of the SPR. Although beaded processes were common, and looked like varicose axons, these processes could always be traced back to a larger parent dendrite. Based on our recent demonstration of massive internalization of the SPR and beading of dendrites of SPR-immunoreactive neurons within 5 minutes of exposure to an *in vivo* injection of SP, we believe that the beading of dendrites corresponds to endosomal loci of recently internalized receptor. Conceivably the dendrites that are beaded in *normal* rats represent sites of internalization of the SPR in response to *endogenous* SP release. Importantly, the beading is found over the entire dendritic surface of the neurons, which suggests that receptors located at both synaptic and nonsynaptic sites internalize in response to SP. These results provide new evidence for functionality of the nonsynaptic SPR in diverse regions of the CNS, and raise the possibility that diffusion of SP to target nonsynaptic SPR is a common feature of CNS neurons.. -The presence of beaded dendrites that extended to the pial surface of the brain tissue (e.g. in the olfactory tubercle) also raises the possiblity that under normal conditions, the receptor can be activated by SP that diffuses through the CSF. It is also possible that SP released from trigeminal primary afferent fibers that innervates dural blood vessels can diffuse to act upon the SPR-immunoreactive dendrites near the pial surface of the brain. Since noxious stimulation evokes the release of SP into the spinal CSF (Go and Yaksh, 1987), neurons with dendrites near the spinal cord surface may be similarly targeted.

The distribution of the SPR and nociceptive processing

The previous ligand binding studies addressed the functional implications of the SPR being found in widely distributed regions of the CNS (Beaujouan et al., 1986; Buck et al., 1986; Quirion et al., 1983; Saffroy et al., 1988; Shults, et al., 1984). We will not

reiterate this discussion. Rather, because of our laboratory's interest in nociceptive processing, we will focus our discussion on the implications of the extensive distribution of the SPR in brain and spinal cord to the contribution of SP to pain generating and control mechanisms at both spinal and supraspinal sites.

Spinal cord and medullary dorsal horns:

There is considerable evidence for a contribution of SP at the level of the spinal cord and medullary dorsal horn. The latter region corresponds to the trigeminal nucleus caudalis. SP is found in small diameter primary afferents that terminate in laminae I and II of the superficial dorsal horn, many neurons of which respond to noxious stimulation (Barber et al., 1979; Hökfelt et al., 1975; Ljungdahl, et al., 1978). As described above noxious stimulation evokes the release of SP in the dorsal horn and in the spinal cord CSF (Duggan et al., 1988) and iontophoretic injection of SP excites dorsal horn nociresponsive neurons (De Koninck et al., 1992; Henry, 1976; Salter and Henry, 1991). Furthermore, intrathecal injection of SP evokes behaviors indicative of pain (Frenk et al., 1988; Papir-Kricheli et al., 1987). The presence of SPR-immunoreactive neurons in laminae I of both the spinal and trigeminal dorsal horns suggests that that there is a direct monosynaptic activation of lamina I nociresponsive neurons by SP-containing primary afferent fibers. As described in chapter VI, we have demonstrated that some of the SPR immunoreactive marginal neurons can be retrogradely labelled from several supraspinal sites, which indicates that these neurons indeed contribute to the rostral transmission of nociceptive messages.

On the other hand, as described in the previous chapters, despite there being a high concentration of SP in the substantia gelatinosa at both the spinal and medullary dorsal horns, we found *no* SPR-immunoreactive neurons in this region; thus the only possible targets of SP released in this region are the dorsally directed dendrites of neurons located ventrally, in lamina III. It is also possible that SP release in the region of the substantia gelatinosa diffuses away from the site of release, to act upon SPR containing neurons located at distant sites, for example, in lamina V. The presence of SPR-immunoreactive

neurons in the region of lamina V of the nucleus caudalis, a region that contains nociresponsive neurons, but a paucity of SP-containing terminals, is consistent with this hypothesis.

Thalamus

There is also evidence that the transmission of nociceptive messages supraspinally involves SP. Several thalamic nuclei that have been implicated in the processing of noccieptive information contained SPR-immunoreactive neurons, including the parafascicular nucleus and several nuclei of the posterior group. Interestingly Battagli and Rustioni recently demonstrated that some SP-containing spinal neurons project to these regions of the thalamus (Battaglia et al., 1992). On the other hand, although these authors also found a projection to the ventrobasal thalamus, we found only very light staining of neurons in the VB. Since there is no established relationship between the density of SPR expression and the functional significance of the SP-SPR receptor interaction, it is difficult to determine the relative importance of SP inputs to the activation of neurons that express the SPR in these different thalamic regions

Although early cytochemical studies found minimal SP terminal immunoreactivity in the thalamus, a reexamination revealed low but detectable SP innervation of the VPL and PO. The depletion of SP in both VPL and PO after cordotomy, which cuts the spinothalamic tract, provided further evidence that a subpopulation of STT axons use SP as a transmitter (Battaglia and Rustioni, 1992). Taken together with the paucity of SP containing terminals in the spinal gray matter ventral to the superficial dorsal horn, these result suggest that the SP-containing spinal projection neurons do not give off significant collaterals in the spinal cord. The paucity of SP terminals around the SPR-immunoreactive neurons in the neck of the dorsal horn is, of course, consistent with the hypothesis that these neurons are targeted by primary afferent derived SP that diffuses after release in superficial dorsal horn.

Parabrachial nucleus

The parabrachial nucleus contained a particularly high concentration of small SPR-immunoreactive neurons, with fine dendritic processes that coursed rostrocaudally. Although this region has traditionally been associated with the transmision of visceral and gustatory inputs, via its connections with nucleus of the solitary tract (Milner and Pickel, 1986), recent studies have established an important contribution of the parabrachial nucleus to nociceptve processing. Specifically, the parabrachial nucleus is a major target of neurons in lamina I, the majority of which respond exclusively to noxious stimulation, and of neurons located in the neck of the dorsal horn and around the central canal (Cechetto et al., 1985; Hylden et al., 1986; Light et al., 1993). Furthermore, many neurons in the lateral parabrachial respond exclusively to noxious stimulation (Bernard and Besson, 1992; Bernard et al., 1994). The latter could be antidromically activated from the central nucleus of the amygdala, providing a disynaptic projection from spinal cord and trigeminal nociceptive responsive neurons to forebrain regions that are implicated in the emotional component of the pain response. Since microinjection of SP in the parabrachial nucleus decreases withdrawal latencies to noxiou heat, it is reasonable to hypothesize the PB neurons that express the SPR are involved in the rostral transmission of nociceptive messages relayed from the spinal cord and trigeminal nucleus caudalis. Whether the SP input to these neurons derives from the latter structures or from local interneurons remains to be determined.

Periaqueductal gray and dorsal raphe

In addition to finding SPR-immunoreactive neurons in "pronociceptive" regions of the spinal cord and brainstem, we found extensive labelling of neurons in regions traditionally associated with antinociceptive controls. For example, both the periaqueductal gray (PAG) and dorsal raphe (DR) of the midbrain contained significant concentrations of SPR-immunoreactive neurons and dendrites; the DR was particularly heavily labelled. Numerous studies have establised the importance of these two regions in the antinociceptive

controls evoked by electirical stimulation or opiate injection (Basbaum and Fields, 1978). There is evidence that these controls are exerted through the activation of descending serotonergic and noradrenergic pathways that inhibit the firing of nociresponsive neurons in the spinal cord (Jensen and Yaksh, 1984; Yaksh, 1979). Since microinjection of SP into the PAG produces antinociception, we have previously raised the possibility that nociceptive input to the midbrain PAG activates descending controls via a SP-containing interneuron (Malick and Goldstein, 1978; Mohrland and Gebhart, 1979). It will be of interest to determine whether the SPR is located on projection neurons that target the cells of origin of the descending monoaminergic pathways, or whether the SPR is expressed by opioid containing interneurons of the PAG.

Locus coeruleus, A5 cell group and the nucleus raphe magnus

We found particularly dense concentrations of SPR containing neurons in several of the pontine noradrenergic cell groups (A5, A6/locus coeruleus). Each of these regions contains spinally-projecting noradrenergic neurons that have been implicated in descending antinociceptive and/or autonomical controls (Proudfit, 1988). Based on studies of Proudfit and colleagues, the possibility that some or all of these systems can be activated via a SP pathway that originates in the nucleus raphe magnus (NRM) has been raised (Yeomans and Proudfit, 1992). In fact, the release of NE in the spinal cord by electrical stimulation of the NRM (Hammond et al., 1985; Sagen and Proudfit, 1987) may involve activation of noradrenergic neurons that express the SPR. We are presently performing double labelling studies to determine the transmitter content of the some of the SPR-immunoreactive neurons in the brainstem.

Although the density of SPR-immunoreactive neurons in the nucleus raphe magnus itself was considerably less than in the noradrenergic cell groups, many neurons were extensively labelled. Their distribution overlapped the distribution of 5-HT-containing neurons in the NRM and adjacent nucleus reticularlis gigantocellularis, pars alpha and their fusiform morphology is similar to that of 5-HT-immunoreactive neurons of these regions

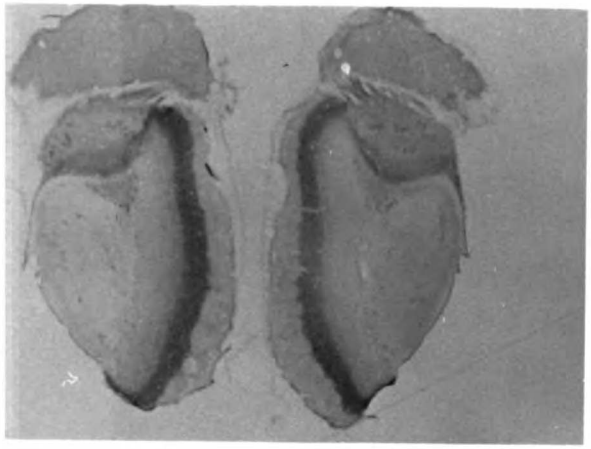
(Bowker and Abbott, 1990; Bowker et al., 1987; Dean et al., 1993; Kwiat and Basbaum, 1990; Kwiat and Basbaum, 1992). Since some SP-containing PAG neurons project to the NRM (Beitz, 1982), it is likely that a SP input to these neurons, at least in part, mediates the activation of raphe spinal neurons by PAG electrical stimulation of opiate injection. Consistent with this hypothesis, iontophoresis of SP excites a subpopulation of neurons in the NRM (Pomeroy and Behbehani, 1980). In future studies, it will be important to determine whether SP microinjection, in fact, produces antinociception and to identify the chemistry and targets of the NRM neurons that express the SPR.

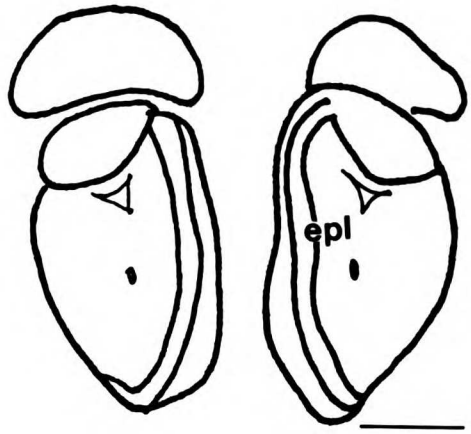
Summary

In summary, we have demonstrated that subpopulations of neurons in functionally distinct regions of the CNS express the substance P receptor over much of their dendritic and somatic surface. Within each brain area, we identified morphologically distinct groups of SPR-immunoreactive neurons. The absence of SPR staining on axons suggests that the SPR is exclusively a postsynaptic target in the CNS. Given the density of SPR on individual neurons and the presence of mismatch between the distribution of SP and the SPR, it is likely that diffusion of SP that acts on nonsynaptic receptors is a common feature of CNS neurons. Given that the SPR is associated with many spinal cord and brainstem loci that have been implicated in the transmission and regulation of nociceptive messages, it is likely that this processes is important in the regulation of pain processing at many levels of the neuraxis.

Figure 1. These photomicrographs illustrate the major loci of SPR immunoreactivity in the rat brain as seen in transverse section. The accompanying schematics are tracings from the photomicrograph and identify the major structures. Abbreviations: epl: external plexiform layer; aon: anterior olfactory nucleus; caudate-putamen (cp); cingulate (cg); triangular septum (ts); paraventricular hypothalamus (pvh); cortical nucleus of the amygdala (coa); medial nucleus of the amygdala (mea); dentate gyrus (dg); habenula (hb); parafascicular nucleus of the thalamus (pf); ventrolateral geniculate (vlg); posterior thalamic nuclei (pt); substantia nigra (sn); superior colliculus (sc); inferior colliculus (ic); periqueductal gray (pag); dorsal raphe (dr); locus coeruleus (lc); pontine reticular nucleus (prn); A5 nucleus (a5); nucleus ambiguus (amg); cuneate nucleus (cun). Calibration bar equals 1.5mm in A; 2.5mm in B; 3mm in C; 3.5mm in E, F, G, H; 2 mm in I, J, L, M; and 1mm in K.

A





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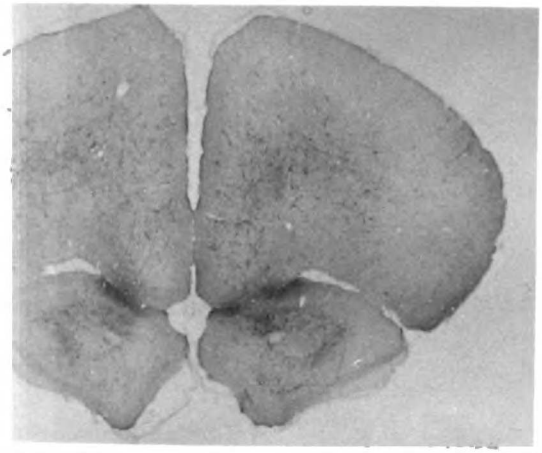

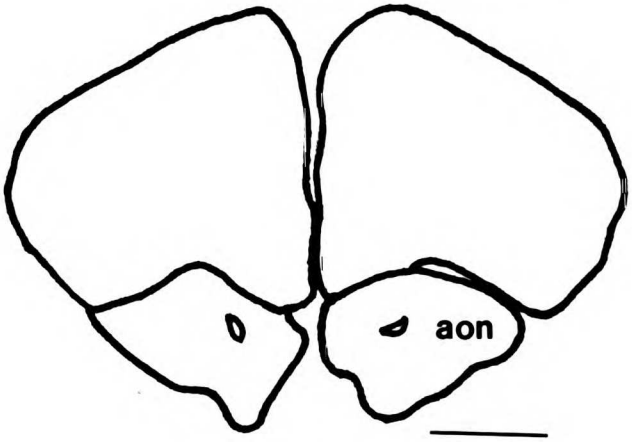

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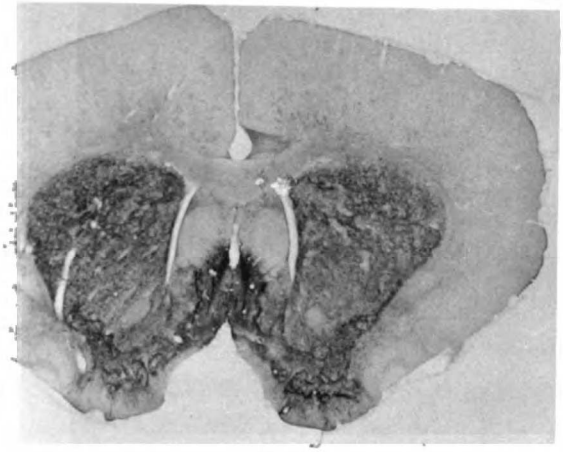

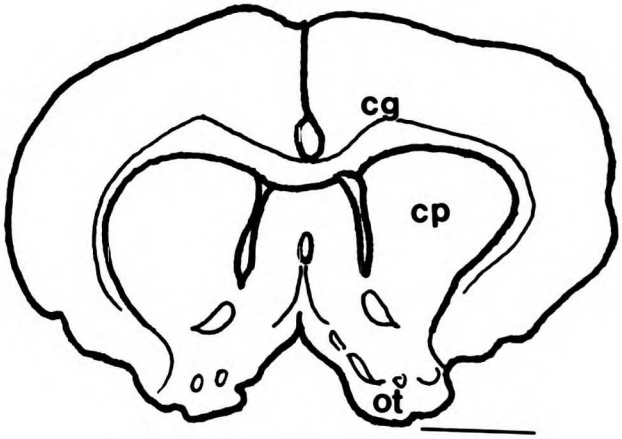

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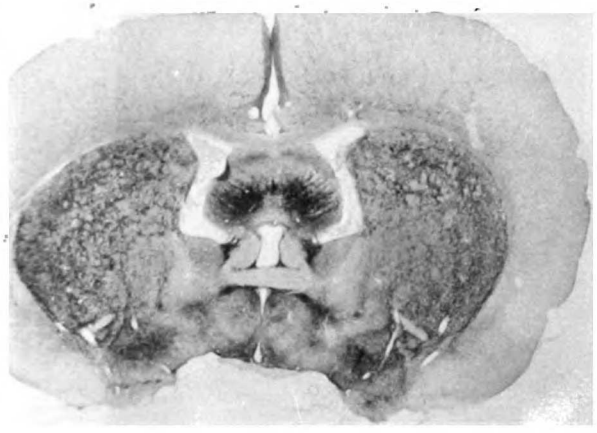

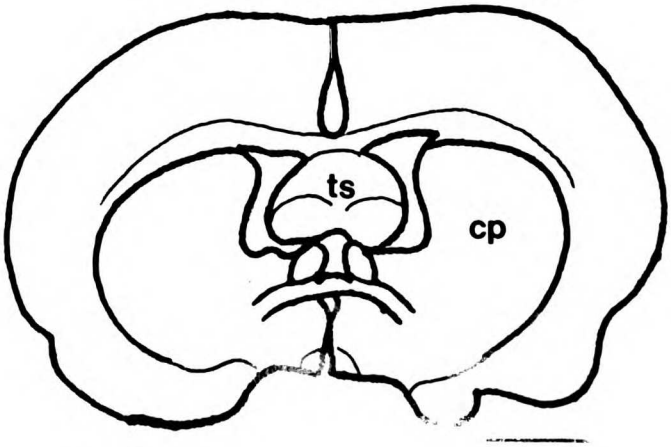

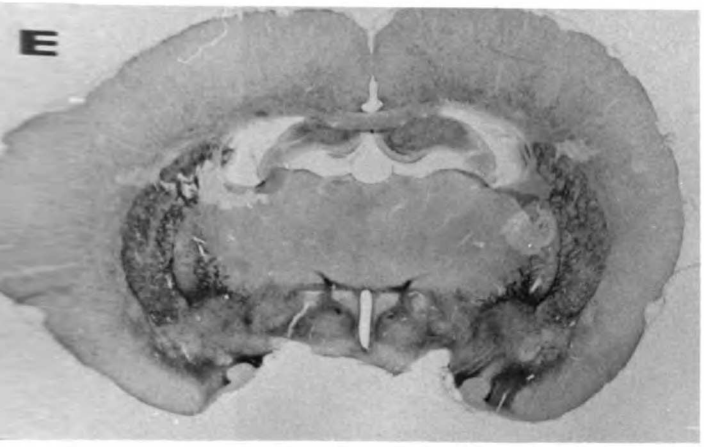
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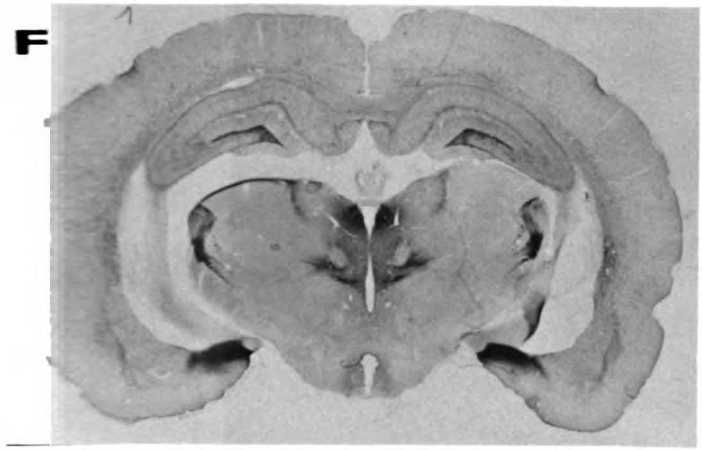
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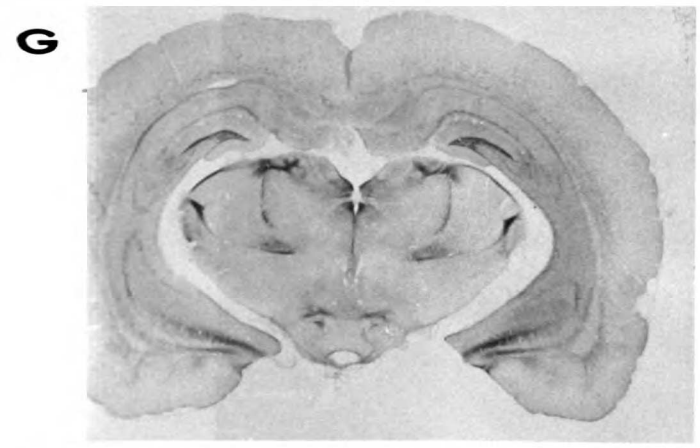
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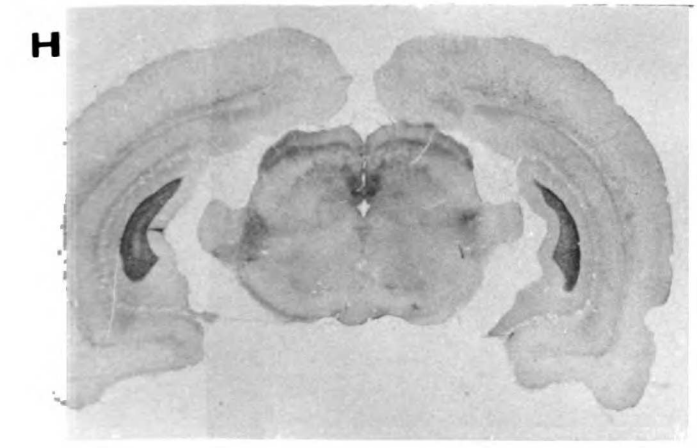
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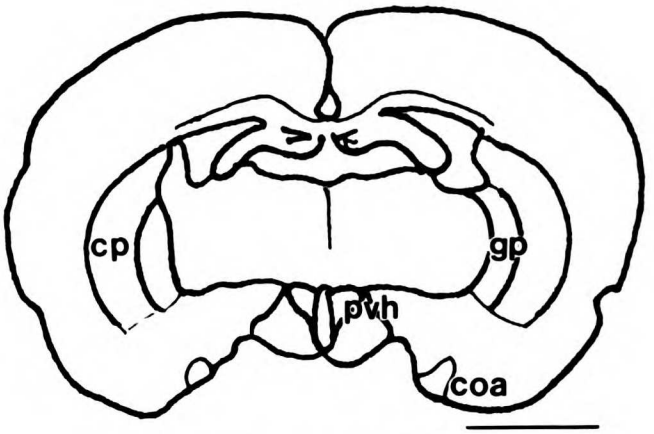
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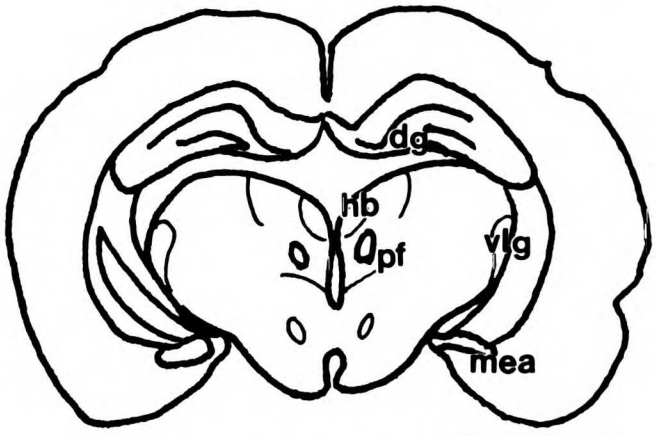
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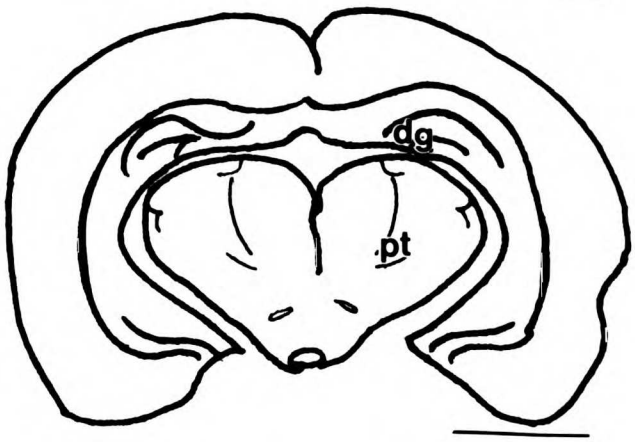
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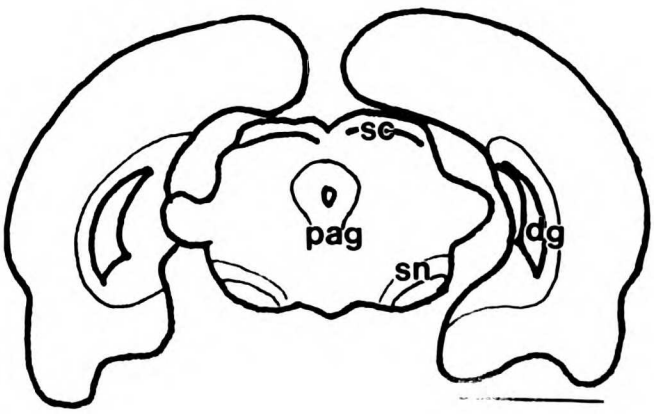
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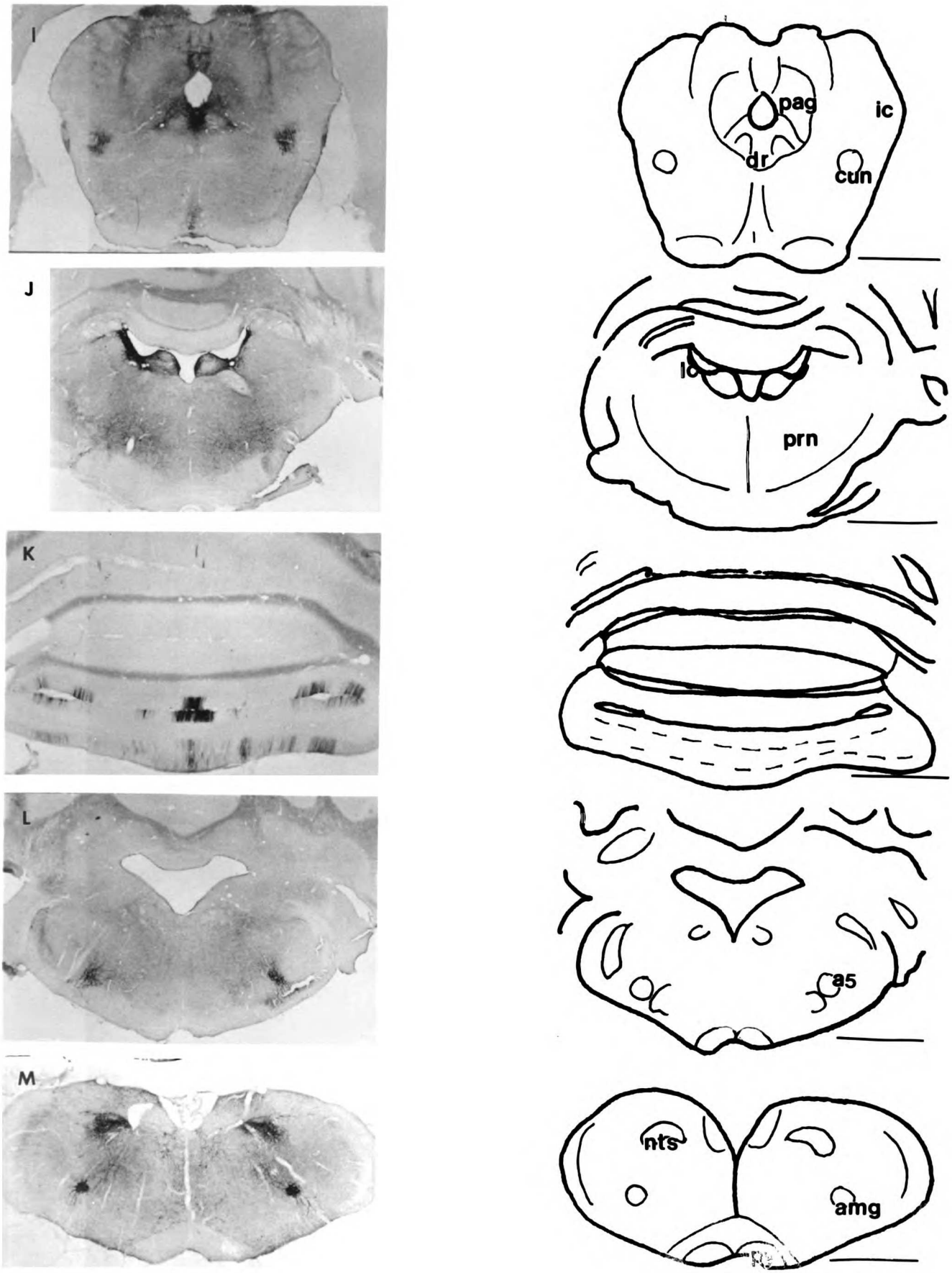
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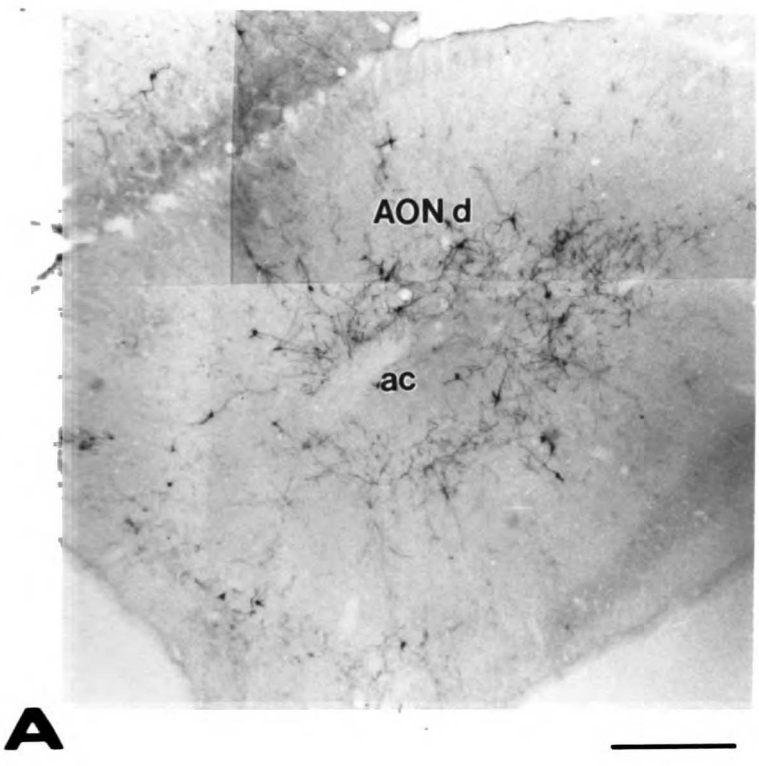
Figure 2. These photomicrographs illustrate the morphology and distribution of SPR-immunoreactivity in several olfactory structures. A: In the anterior olfactory nucleus (AON) surrounding the anterior commissure (ac), we recorded a discrete-population of SPR-immunoreactive neurons. B: Higher magnification of the pars dorsalis of the AON shows the morphology of the labelled neurons. C: Note the diffuse cloud of SPR immunoreactivity in the external plexiform layer (epl), within which individual neurons could not be discerned. The ependymal zone (ez) contains a small number of labeled neurons seen in the ependymal zone (ez). Calibration bars equals 350 μm in A and 50 μm in B, C.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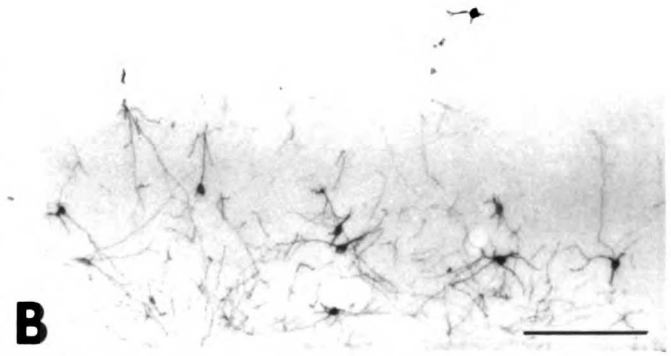
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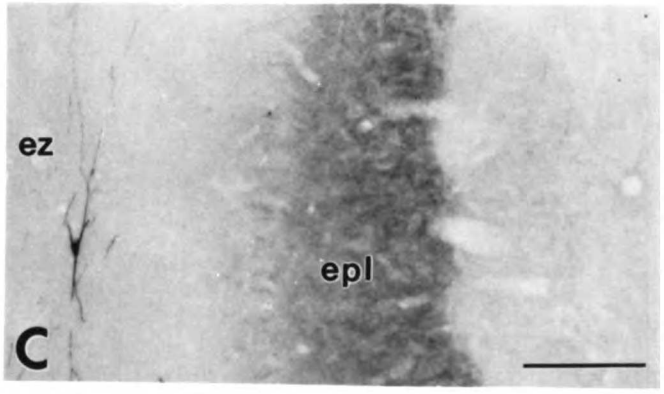
ez
epl
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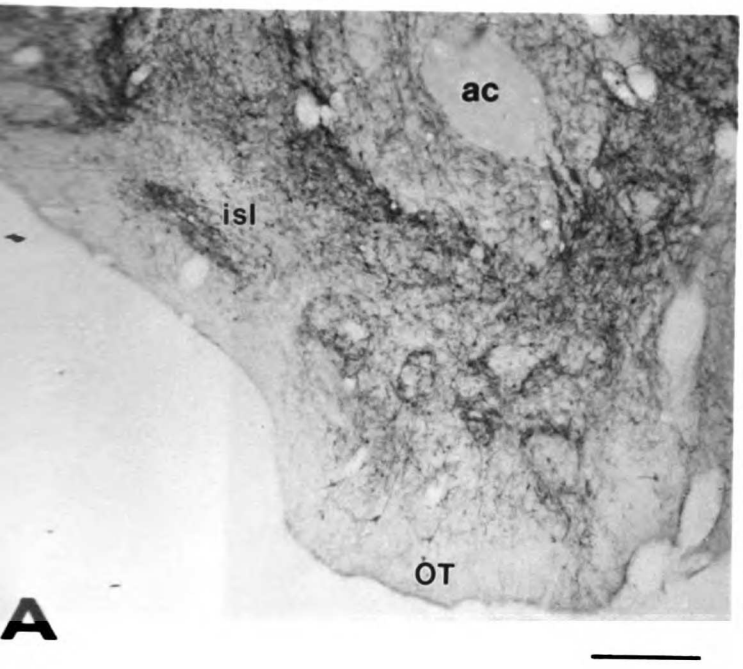
Figure 3. A: The superficial layers of the olfactory tubercle (OT) contain very densely stained SPR-immunoreactive neurons; clusters of labeled neurons were noted within the islands of Calleja (isl). B: The nucleus accumbens (ACB), adjacent to the anterior commissure (ac), contains large numbers of SPR-immunoreactive neurons; their fusifom morphology and the presence of varicose dendrites (arrowhead) can be seen in C. Calibration bars equals 400 in A, B and 100 μm in C.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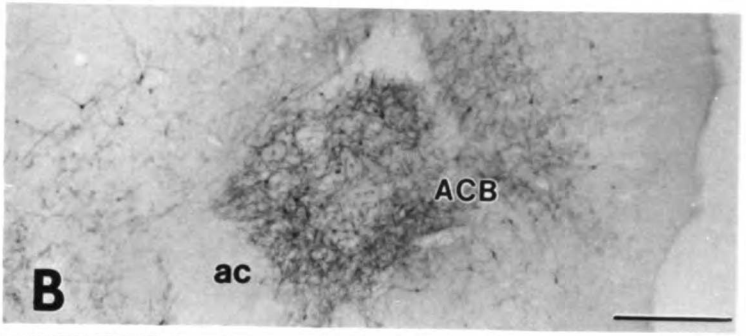
ACB
ac
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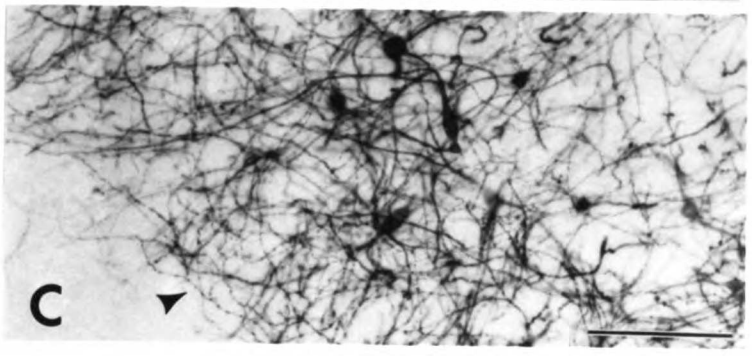
C

Figure 4. These photomicrographs illustrate SPR-immunoreactive neurons in the superficial and deep laminae of the cerebral cortex. A: Lightly labeled multipolar neurons are characteristic of lamina II of the cerebral cortex. B: Double-bouquet neurons are common in the superficial cortical laminae. C: Occasionally, we observed SPR-immunoreactive pyramidal neurons. D and E: SPR-immunoreactive neurons were clustered in the deep lamina VI, surrounding the cingulum bundle (cg). F: Both multipolar and fusiform SPR-immunoreactive neurons were located in the deep cortical laminae (VI and ventralmost portion of V). G: Many of the dendrites arising from lamina VI neurons were highly varicose (arrowhead). Calibration bars equal 150 μm in A, B, C; 300 μm in D; 200 μm in E and 150 μm in F, G.

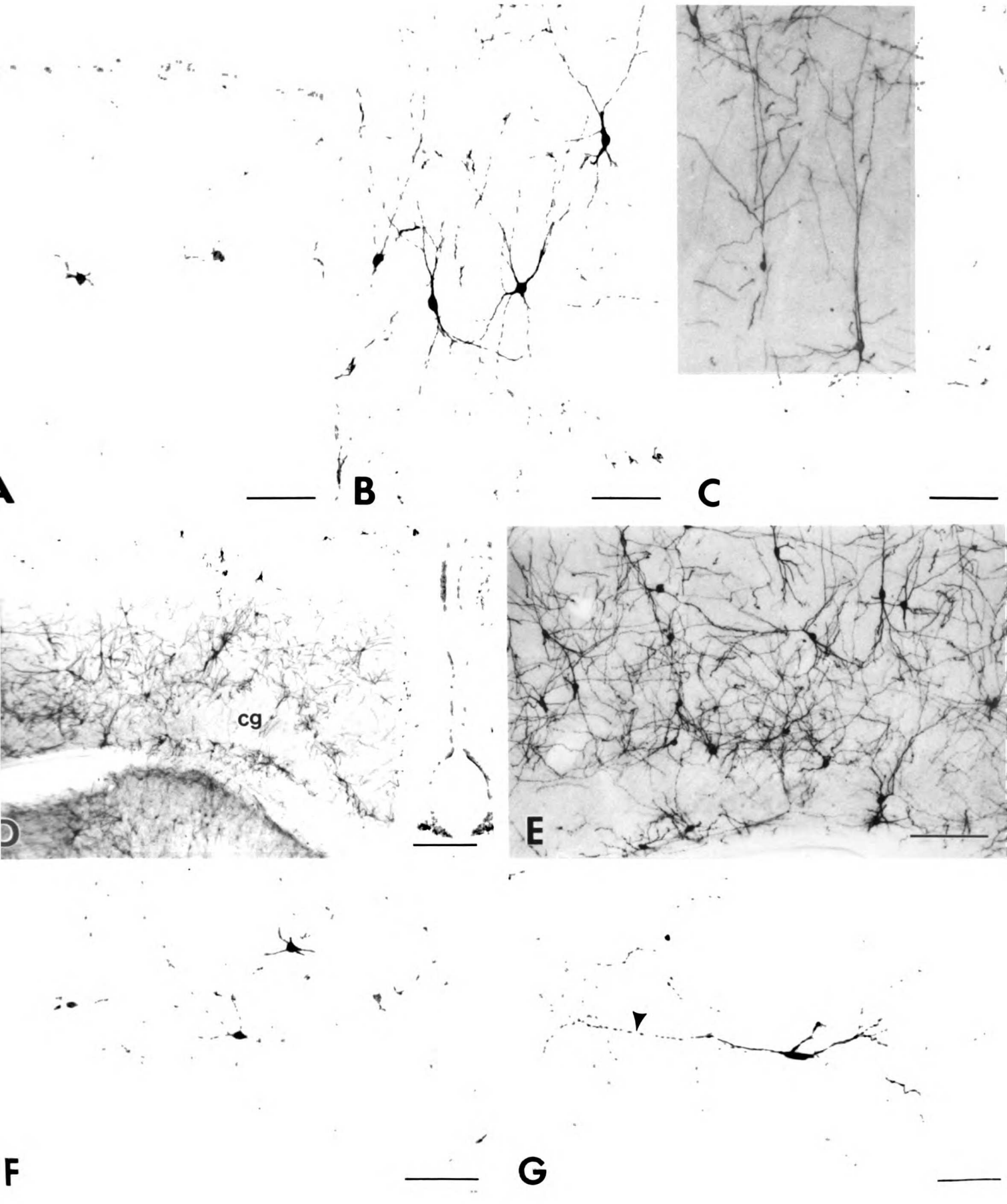
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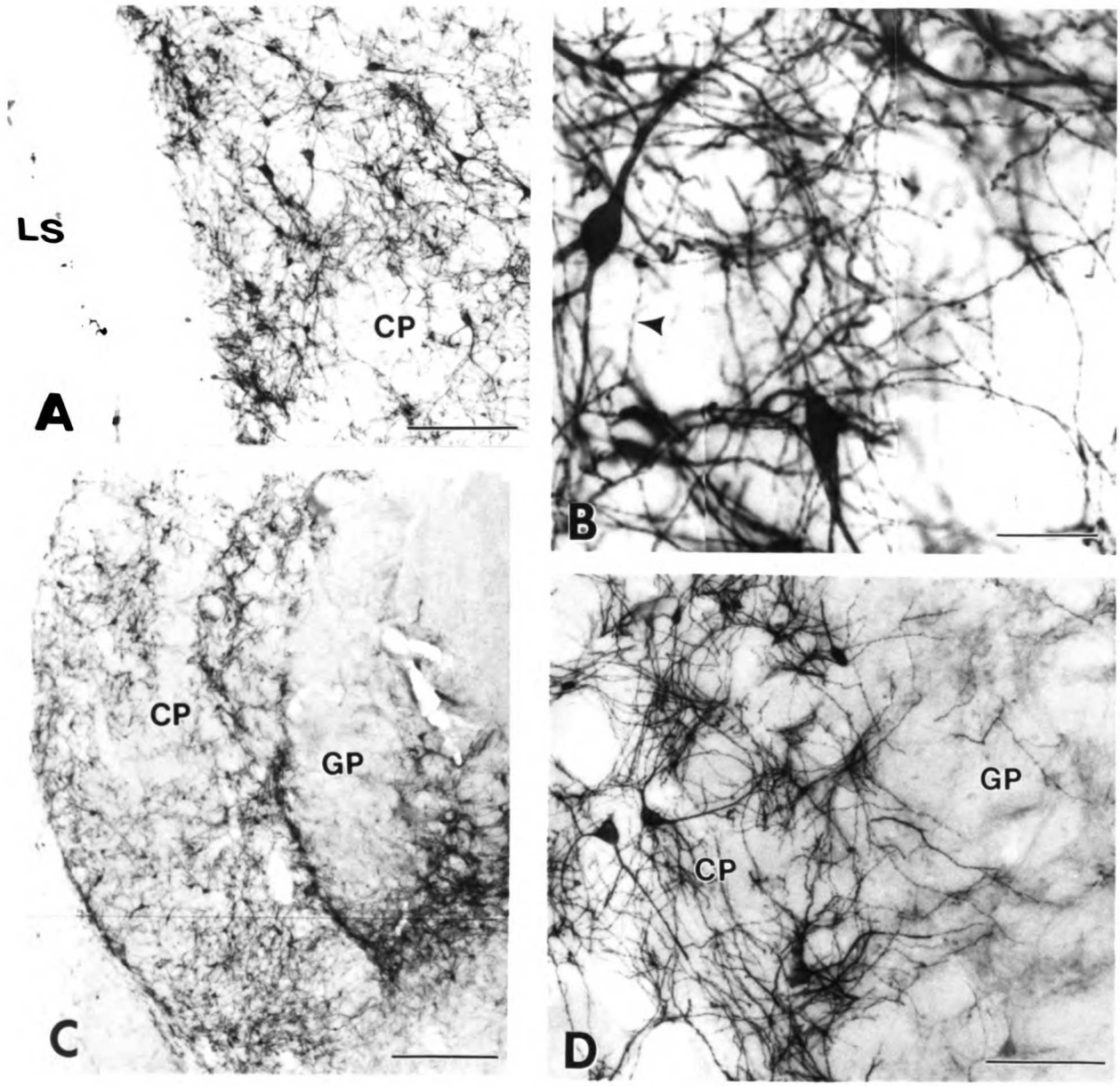
Figure 5. The caudate-putamen (CP) contains a dense meshwork of SPR-immunoreactivity; the density of staining is much less in the globus pallidus (GP). A: A: This section is located ajacent to the lateral septum (LS) and illustrates the clusters of labelled cell bodies and dendrites in the caudate-putamen (CP) B: SPR-immunoreactive neurons in the CP have fusiform cell bodies with both smooth and beaded dendrites (arrowhead). C,D: These figures illustrate the difference in staining pattern in the CP and GP. Calibration bars equal 200 μm in A; 50 μm in B; 400 μm in C; and 100 μm in D.


LS
CP
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GP
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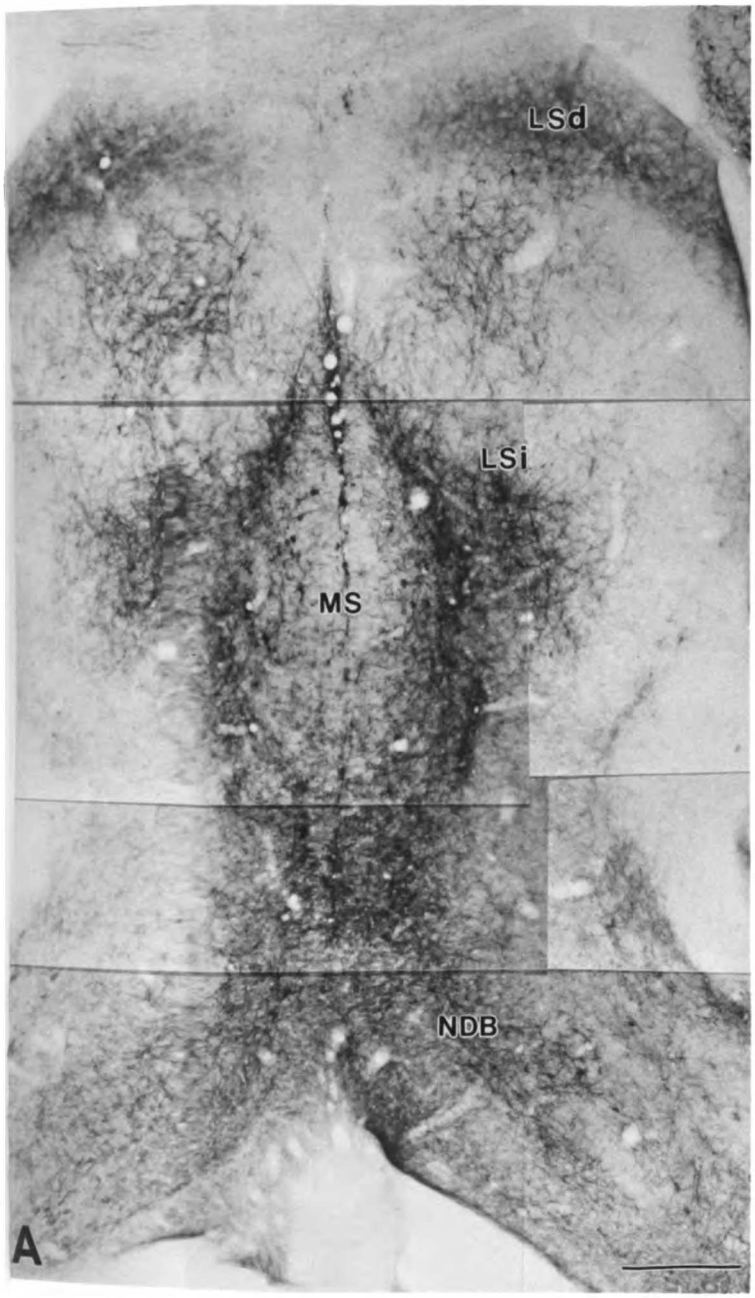
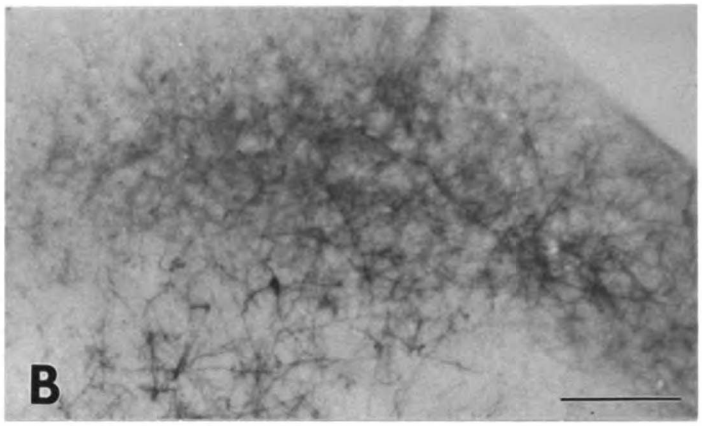
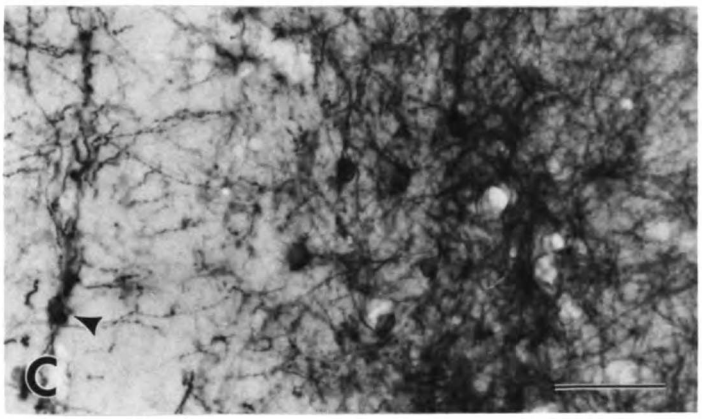
D

Figure 6. The photomontage (A) and accompanying high power photomicrographs illustrate the distribution and different densities of SPR immunoreactivity in the septal complex and in the diagonal band. B: The lateral septal nucleus (LSd) contains diffuse SPR immunoreactivity.with isolated small neurons. C: The medial septal nucleus (MS) contains a much denser pattern of staining, with large, round heavily labeled neurons (arrowhead). Beaded processes were common. D: The nucleus of the diagonal band (NDB) contained smaller cell bodies and many thin, highly varicose dendrites (arrowhead). Calibration bars equals 400 μm in A; 50 μm in B and 100 μm in C,D.


LSd
LSi
MS
NDB
A


B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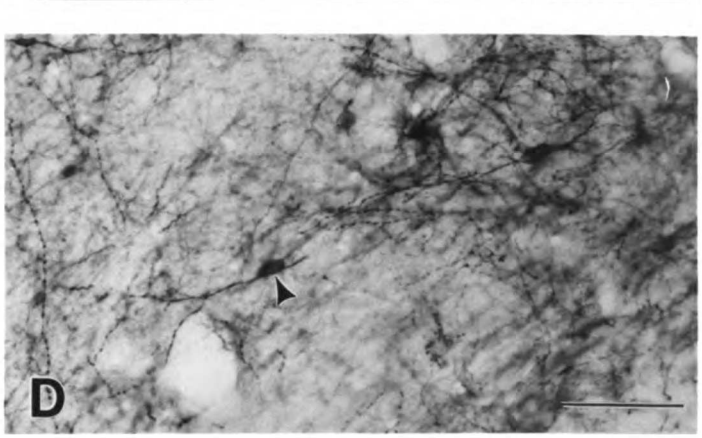

D

Figure 7. This photomontage (A) and the accompanying high power photomicrographs illustrate the distribution and different densities of SPR immunoreactivity in the hippocampal formation. C: The dentate gyrus, in fact, contains the highest density of SPR-immunoreactive neurons. They are heavily lablled and have thick dendrites. The CA1 field contains a sparse population of lightly labeled neurons in the pyramidal layer. Ventral to the pyramidal labeling, denser SPR immunoreactive neurons can be seen seen along the hilar fissure. B: Higher magnification of the CA1 field illustrates the lightly labelled cells in the pyramidal layer (arrowhead) and the thin band of staining just dorsal to the hilar fissure (arrow). A few very lightly labelled neurons, with a basket cell morphology can be seen immediately dorsal to a region of dense labelling in the dentate gyrus (arrowhead). D: Higher magnification shows the morphology of the densely labeled neurons found in the fasciola cinerea component of the subiculum. Calibration bars equal 400 μm in A; 125 μm in B; 100 μm in C and 150 μm in D.

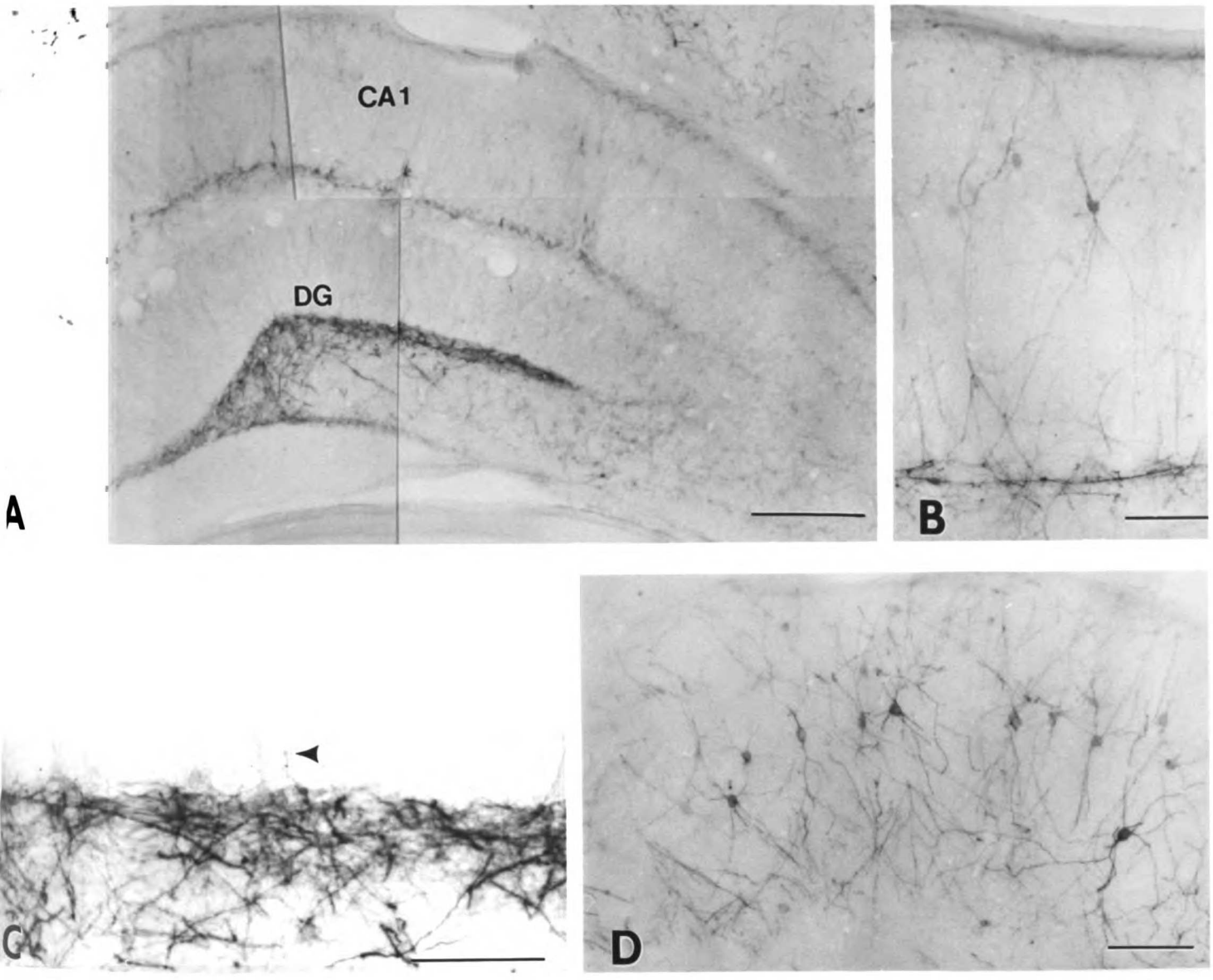

CA1
DG
A
B
C
D

Figure 8. These photomicrographs illustrate the distribution of SPR immunoreactivity in different regions of the the amygdaloid complex. A: The medial nucleus of the amygdalacontains a very diffuse, cloud of immunoreactivity; this staining pattern continues caudally into the posterior nucleus of the amygdala. Fine processes located around the edges of the dense staining suggest that the overall diffuse quality results from a light labeling of fine dendrites. B: Bands of labeled processes (presumed dendrites) are present in the cortical amygdaloid nucleus; these can be appreciated better in C. D: The basolateral nucleus (BLA) and the central nucleus (CEA) are almost devoid of SPR immunoreactivity, but are outlined by staining that surrounds them. Some patches of staining are found in the CEA. Calibration bars equal 200 μm in A and C; 400 μm in B and 300 μm in D.

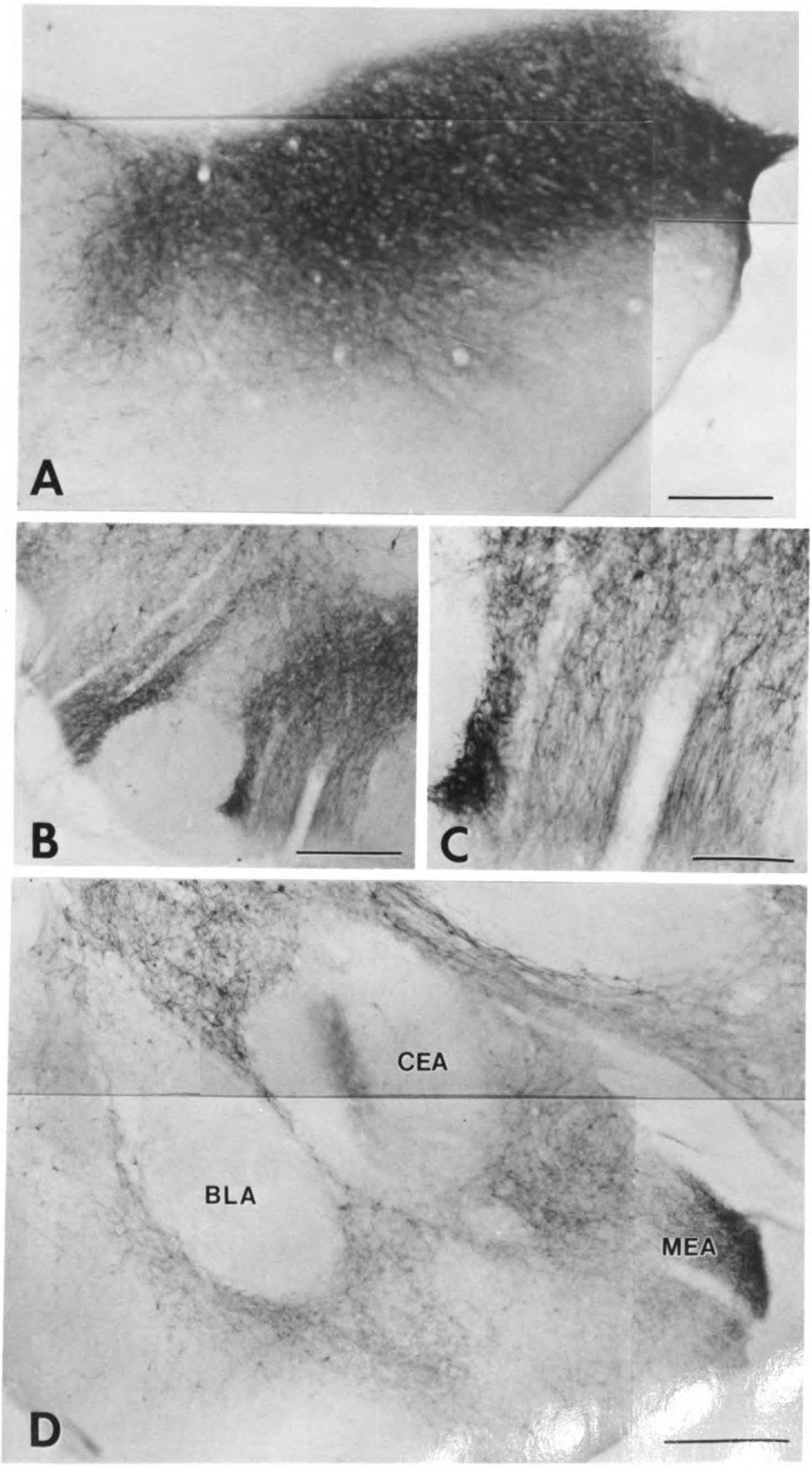

A
B
C
CEA
BLA
MEA
D

Figure 9. These photomicrographs illustrate examples of the distribution of SPR immunoreactivity in the hypothalamus. A: There is dense, but diffuse immunoreactivity surrounding the supraschiasmatic nucleus (SCH). Dorsal to the fornix (fx) and ventral to the anterior thalamic nuclei is a band of immunoreactivity that extends dorsolaterally. B: There is very light staining in the anterior hypothalamic nucleus (AHN); the intensity of the staining increases in the regions adjacent to the third ventricle (V3) and surrounding the paraventricular nucleus (PVH), which itself is not labelled. The median eminence (ME) contains a few very large SPR-immunoreactive neurons. Calibration bars equal 300 μm in A, B.

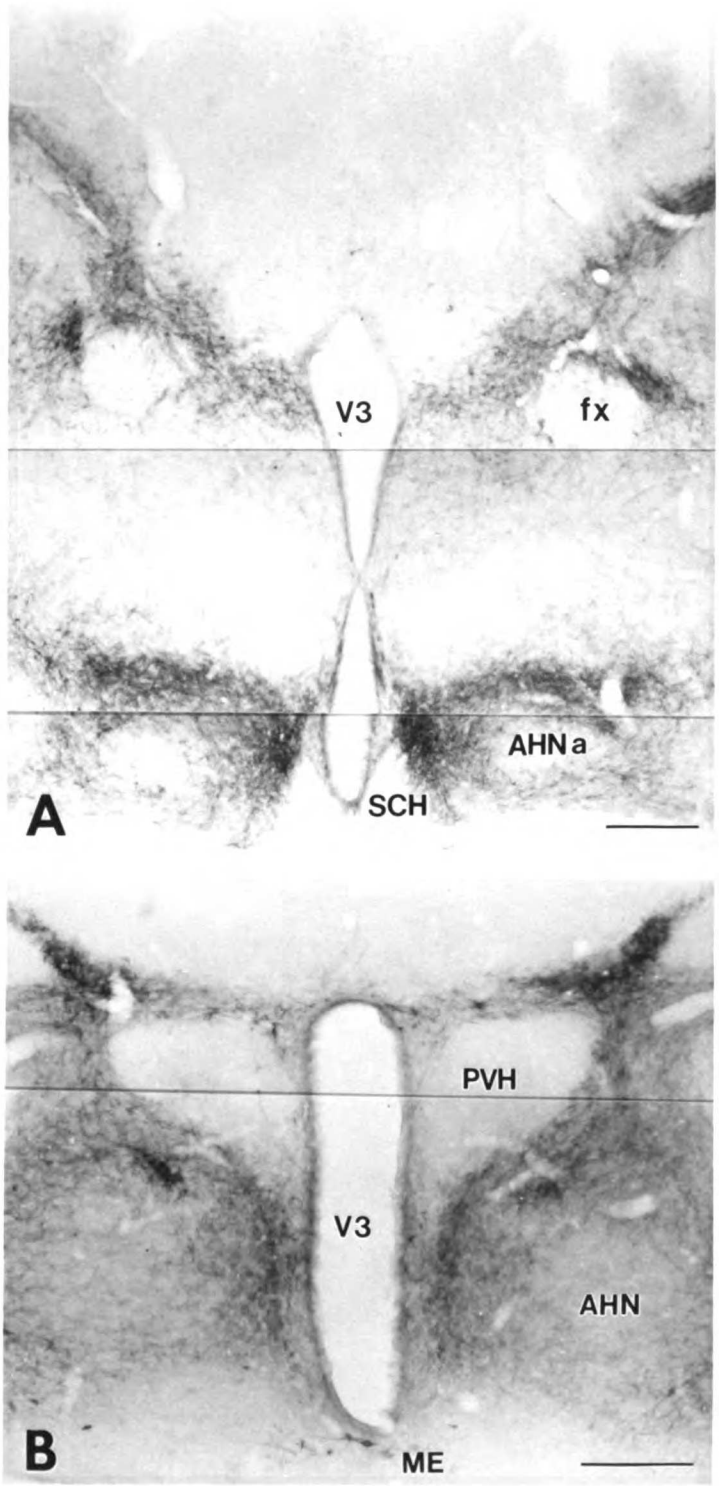
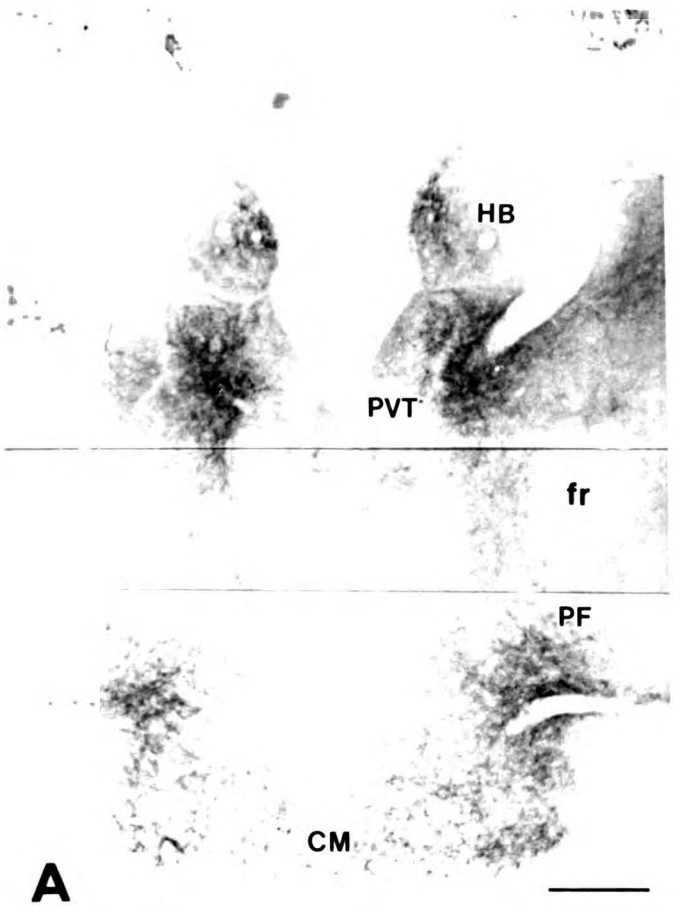
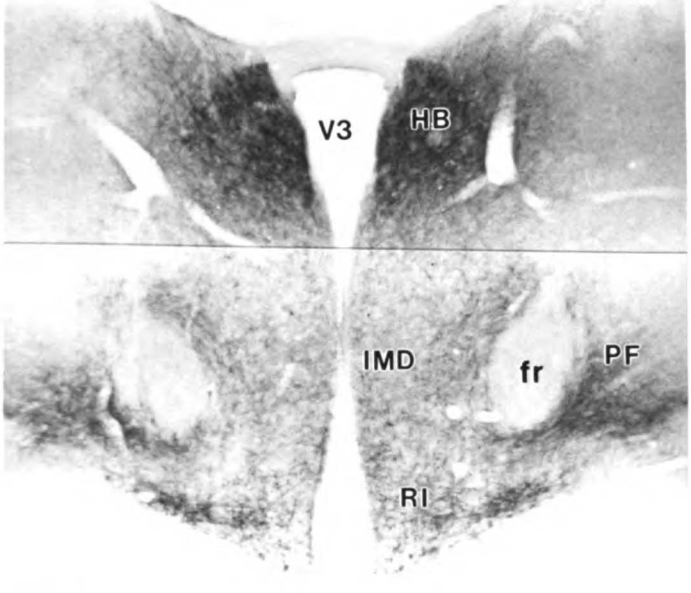

V3
fx
AHNa
SCH
A
PVH
V3
AHN
B
ME

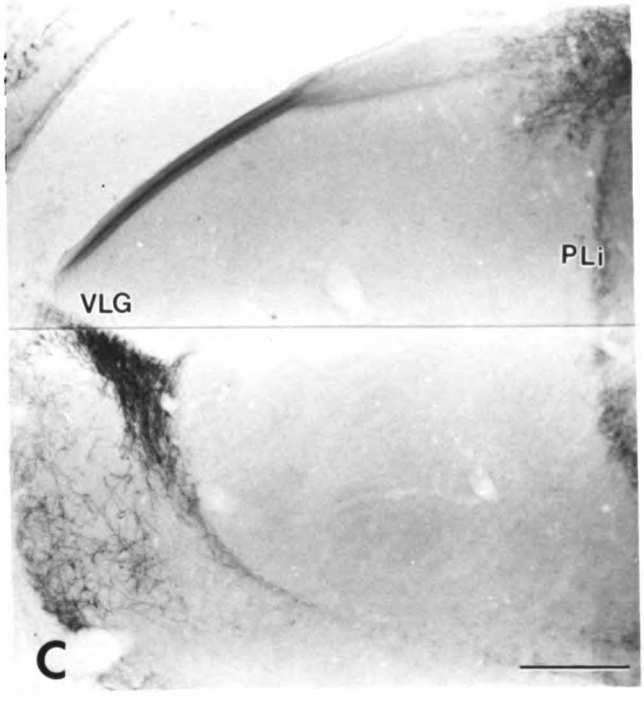
Figure 10. These photomicrographs illustrate areas of SPR immunoreactivity in the thalamus. A: There are moderate levels of SPR immunoreactivity in the medial habenula (HB) and in the area surrounding the paraventricular nucleus of the thalamus (PVT). The PVT contains little staining. There is diffuse staining around the fasicuclus retroflexus (fr), in the area of the parafascicular nucleus (PF) and in the central medial nucleus (CM). B: The density of staining increased more cauadally, notably in the habenula and in the parafascicular nucleus. Moderate SPR immunoreactivity was also seen in the intermediodorsal nucleus (IMD) and in the area of the rostral interstitial nucleus (RI). C: This montage shows SPR immunoreactivity at more posterior levels of the thalamus. There is very dense staining in the ventrolateral geniculate (VLG) and a moderate band of SPR immunoreactivity in the area of the posterior limitans nucleus (PLi). D: Higher magnification of the VLG shows the dense concentration of SPR immunoreactive cell bodies and dendrities; the medial parvocellular portion of the VLG contains the most intense staining. Calibration bars equal 400 μm in A and B; 300 μm in C and 150 μm in D.



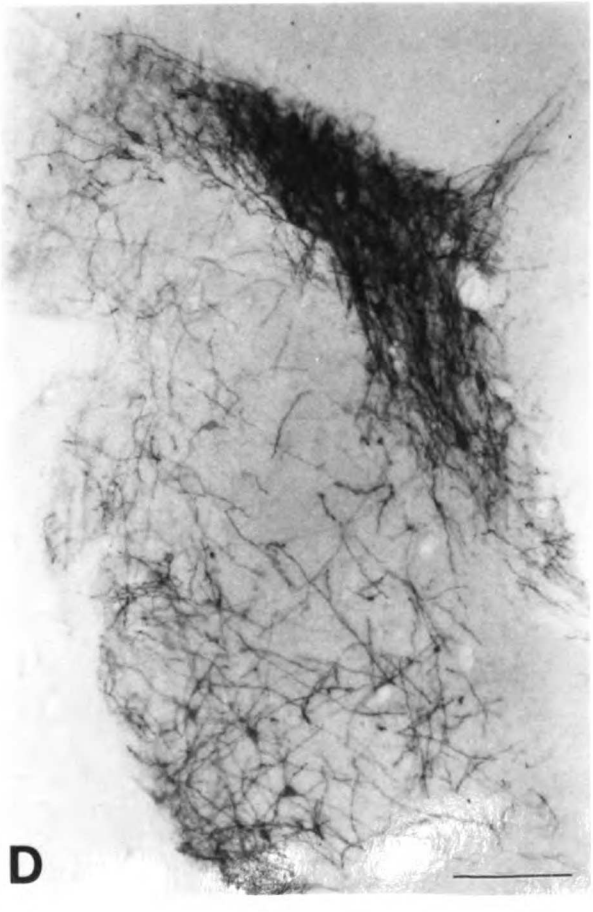
A



B



C



D

Figure 11. These photomicrographs illustrate the pattern of SPR immunoreactivity in the periqueductal gray (PAG) at the level of the superior colliculus. A: The staining is concentrated in a relatively homogeneous population of neurons in the dorsal and ventrolateal PAG, from the aqueduct (aq) to the lateral borders of the PAG. B: A very dense meshwork of labelled dendrites is seen at the level of the posterior commissure. A few labelled dendrites course dorsally through the commissure. C: Even more intense staining is found in the dorsal raphe nucleus, in the ventral part of the PAG. Calibration bars equal 50 in A,B,C.

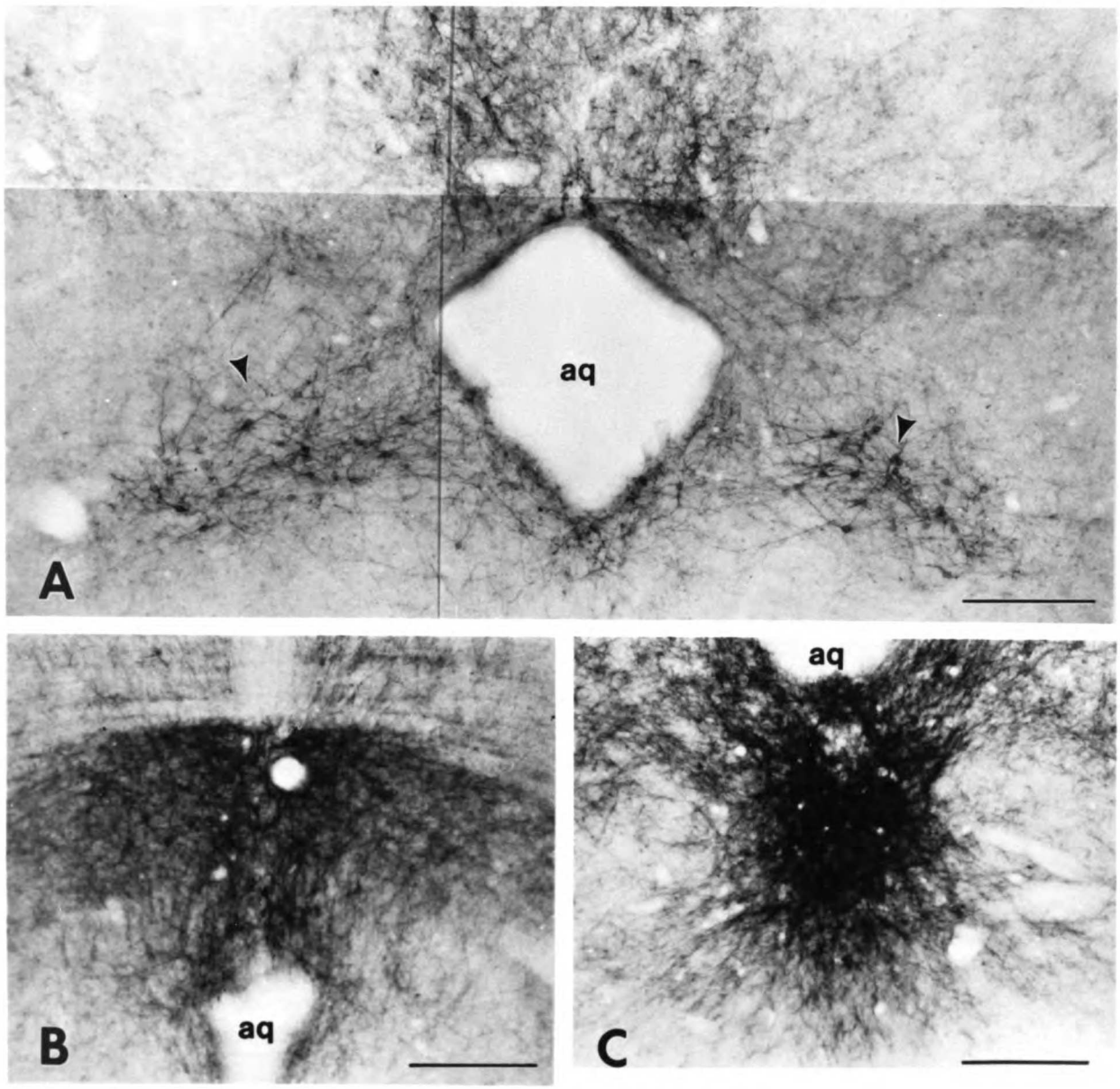

aq
A
B
aq
aq
C

Figure 12. Particularly dense staining is found in the in the parabrachial nucleus (PB) and the locus coeruleus (LC) of the dorsolateral pons. A: Transverse section of the PB shows the dense SPR immunoreactivity in both the dorsolateral and medial parabrachial nuclei, which surround the brachium conjunctivum. B: The fact that the SPR immunoreactivity is located on fine processes that course in the rostral caudal plane can be appreciated in sagittal section of the PB C: Transverse section of the LC, adjacent to the fourth ventricle (V4) illustrates that the very dense concentration of SPR immunoreactivity in this region makes identification of labelled cell bodies difficult. D: As in the sagittal section through the LC illustrates that the majority of SPR immunoreactivity is localized to fine processes (arrowhead) directed rostrocaudally. Calibration bar equals 50 μm in A-D.

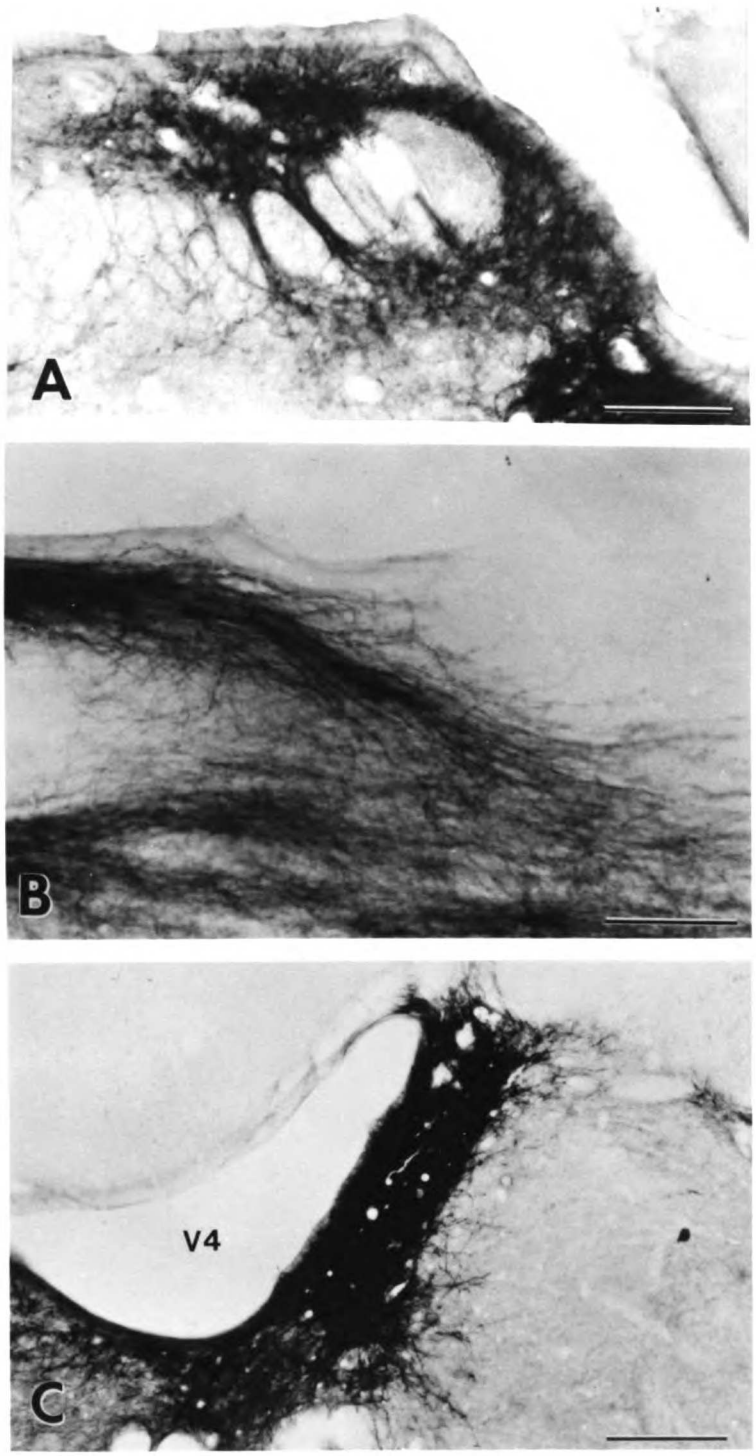

A
B
V4
C

Figure 13. This photomicrograph of a transverse section through the cerebellum illustrates that the staining is associated with the dense stripes of SPR immunoreactivity that are concentrated in molecular layer (mol). Lighter staining is also seen in the white matter ajacent to the granule cell layer (gr). Calibration bar equals 100 μm.

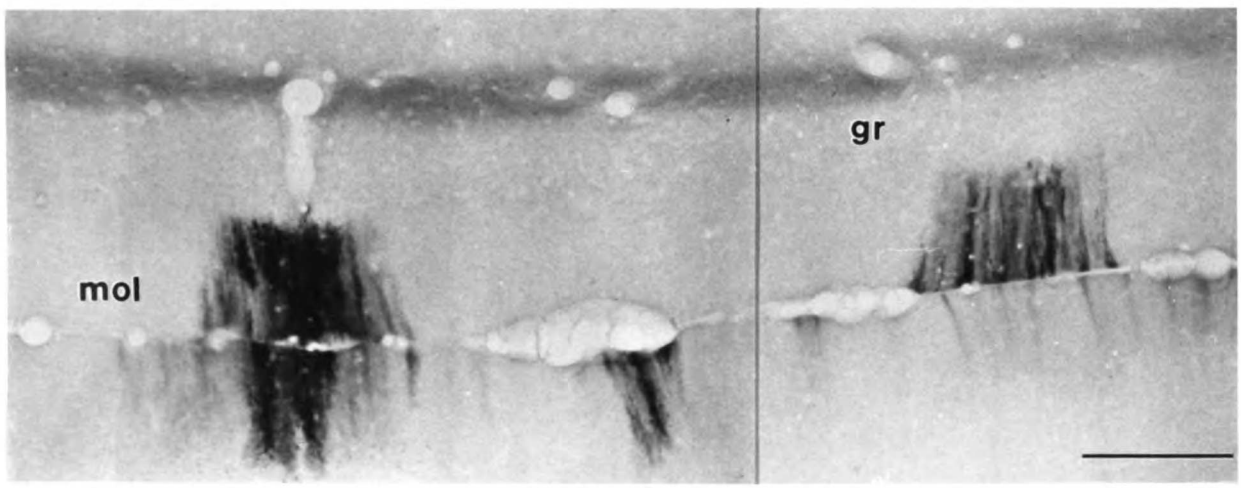
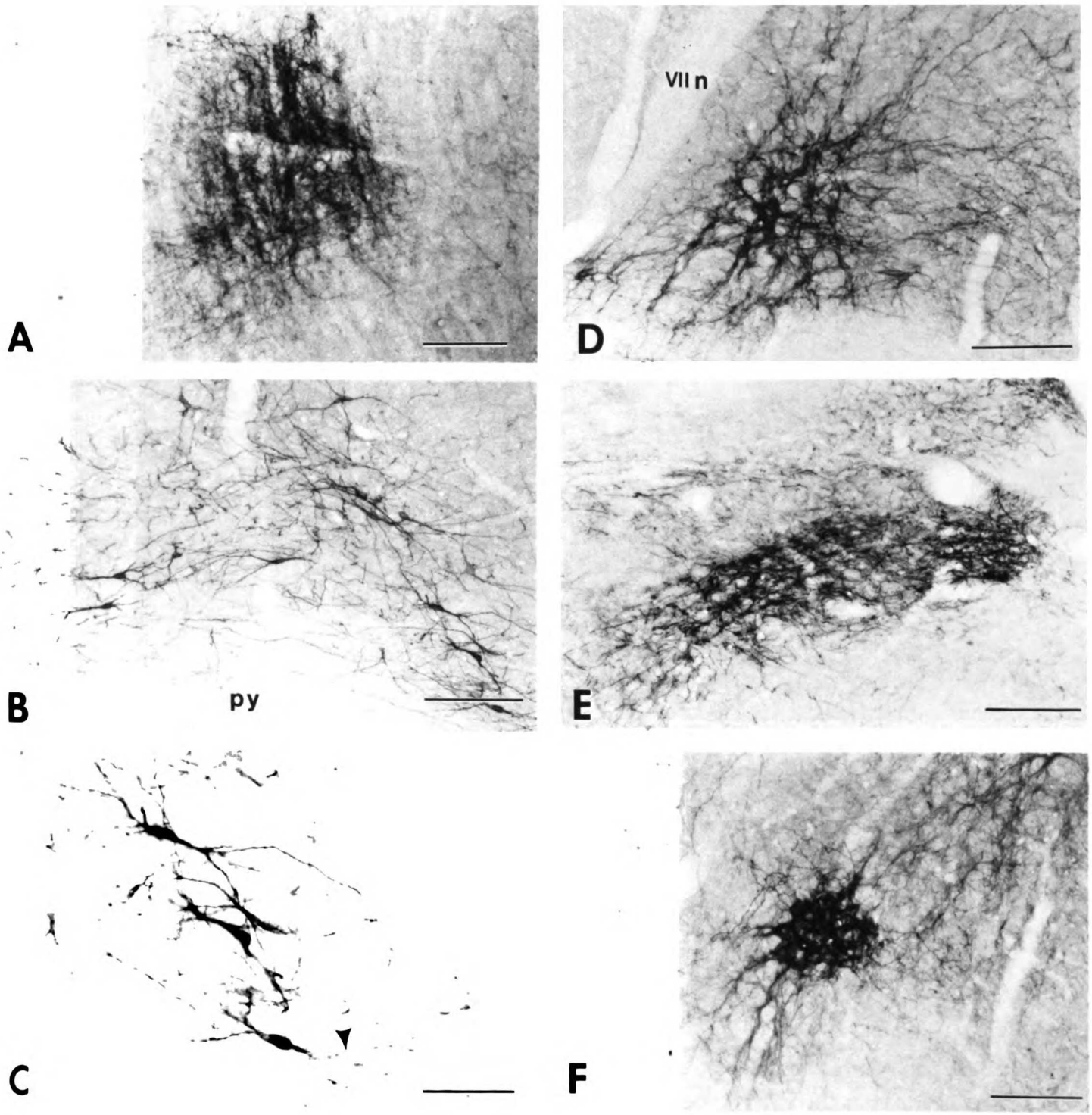

mol
gr

Figure 14. These photomicrographs show the foci of SPR immunoreactivity in several brainstem regions. A: The nucleus cuneformis, at the level of the inferior colliculus, contains densely packed with SPR-immunoreactive cell bodies and dendrites. B: The nucleus raphe magnus (NRM), at the level of the VIIth nucleus, contains scattered, but well labelled SPR-immunoreactive neurons; py: pyramids. Most of the NRM neurons are large and fusiform, with dendrites that arborize mediolaterally; many of the latter are beaded. D: The A5 nucleus, located medial to the VIIth nerve (VII n) contains very heavily labelled SPR-immunoreactive neurons; the medially adjacent superior olive is devoid of immunoreactivity. E: This micrograph shows the dense distribution of SPR immunoreactivity in the nucleus of the solitary tract, at a level rostral to the obex. F: The nucleus ambiguus contains very heavily labelled SPR-immunoreactive neurons. Calibration bars equal 50 μm in A,B,D,E,F; and 100 μm in C.


A
B
py
C
D
VII n
E
F

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CHAPTER IV

Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis

Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis

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Running Title: Substance P receptor in rat spinal cord.

Number of illustrations: 9

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ABSTRACT

Although there is considerable evidence that primary afferent-derived substance P contributes to the transmission of nociceptive messages at the spinal cord level, the population of neurons that express the substance P receptor and thus are likely to respond to substance P has not been completely characterized. To address this question, we used an antibody directed against the C-terminal portion of the rat substance P receptor to examine the cellular distribution of the receptor in spinal cord neurons. In a previous study, we reported that the substance P receptor decorates almost the entire dendritic and somatic surface of a subpopulation of spinal cord neurons. In the present study we have taken advantage of this labelling pattern to identify morphologically distinct subpopulations of substance P receptor-immunoreactive neurons throughout the rostral-caudal extent of the spinal cord. We observed a dense population of fusiform substance P receptor-immunoreactive neurons in lamina I at all segmental levels. Despite having the highest concentration of substance P terminals, the substantia gelatinosa (lamina II) contained almost no substance P receptor-immunoreactive neurons. Several distinct populations of substance P receptor-immunoreactive neurons were located in laminae III- V; many of these had a large dorsally directed dendritic arbor that traversed the substantia gelatinosa to reach the marginal layer. Extensive labelling was also found in neurons of the intermediolateral cell column. In the ventral horn, we found that labelling was associated with clusters of motoneurons, notably those in Onuf's nucleus in the sacral spinal cord. Finally, we found no evidence that primary afferent fibers express the substance P receptor. These results indicate that relatively few, but morphologically distinct subclasses, of spinal cord neurons express the substance P receptor. The majority, but not all of these neurons, are located in regions that contain neurons that respond to noxious stimulation.

TABLE OF ABBREVIATIONS

5-HT	serotonin
CA	central autonomic nuclei
CGRP	calcitonin gene-related peptide
CSF	cerebrospinal fluid
DC	dorsal column white matter
DRG	dorsal root ganglion
IML	intermediolateral cell column
LMN	latero-medial nucleus of ventral horn
LSN	lateral spinal nucleus
mRNA	messenger RNA
NK-1	neurokinin-1 receptor
NK-2	neurokinin-2 receptor
NK-3	neurokinin-3 receptor
NKA	neurokinin A
PB	phosphate buffer
PBS	phosphate-buffered saline
SG	substantia gelatinosa
SP	substance P
SPN	sympathetic preganglionic neuron
SPR	substance P receptor
TNC	trigeminal nucleus caudalis
VMN	ventromedial nucleus of ventral horn

INTRODUCTION

The undecapeptide substance P (SP), a member of the tachykinin neuropeptide family, is an important signalling molecule that has been implicated in several functionally distinct spinal cord systems. In addition to a critical contribution to the transmission of nociceptive messages by primary afferents, SP regulates the outflow of both ventral horn motoneurons and preganglionic sympathetic neurons of the intermediolateral cell column (IML). The evidence for these diverse actions of SP comes from anatomical, electrophysiological and pharmacological studies. In brief, substance P is synthesized by small diameter, primary afferent fibers, many of which respond to noxious stimulation and terminate in regions of the spinal cord dorsal horn that contain neurons responsive to noxious stimulation, particularly the superficial laminae I and II (Barber et al., 1979; Hökfelt et al., 1975; Ljungdahl et al., 1978). A much smaller SP terminal field is located in the region of lamina V, which also contains nociresponsive neurons (De Koninck et al., 1992; Hökfelt, et al., 1975; Menétrey et al., 1977). Whether the peptide in lamina V derives from primary afferents is not clear; it may arise from SP-containing neurons that are intrinsic to the dorsal horn, and which have been demonstrated after colchicine treatment, or by *in situ* hybridization for the preprotachykinin mRNA precursor of SP.

Most importantly, noxious mechanical stimulation of skin and electrical stimulation of peripheral nerve at C fiber strength evokes the release of SP in the spinal cord cerebrospinal fluid (CSF) (Duggan et al., 1988); the extent to which noxious heat stimulation evokes SP release is not clear (Kuraishi et al., 1989). Other studies suggest that prolonged and intense cutaneous stimulation, which results in tissue damage and inflammation, is necessary for the release of SP. Iontophoresis of SP also selectively excites nociresponsive neurons in the spinal dorsal horn; there is minimal effect on non-nociresponsive neurons (De Koninck and Henry, 1991; Henry, 1976; Salter and Henry, 1991); however, see (Fleetwood-Walker et al., 1990; Fleetwood-Walker et al., 1993). *In vitro* studies demonstrated that SP induces a delayed, but prolonged depolarization of dorsal horn neurons and can increase the excitability of neurons, in

part by enhancing NMDA-mediated glutamatergic effects (Randic et al., 1990; Rusin et al., 1992). Finally, intrathecal administration of SP evokes a morphine sensitive "pain" behavior, including scratching of the skin and vocalization (Randic, et al., 1990; Rusin, et al., 1992). To what extent this represents pain behavior has, however, been questioned (Cridland and Henry, 1988; Hylden and Wilcox, 1981; Larson, 1988). Finally, there is evidence that the excitatory effect of SP on dorsal horn firing and on behavior (Yashpal et al., 1993) can be blocked by selective SPR, also known as neurokinin 1 (NK-1) antagonists (vide infra).

Substance P has also been implicated in the regulation of ventral horn motoneuron function. Immunohistochemical studies revealed a dense innervation of spinal motoneurons by substance P-containing terminals, many of which cocontain serotonin and arise from SP-containing neurons of the ventral medulla (Helke et al., 1982; Menétrey and Basbaum, 1987; Reddy et al., 1990). In fact, neurotoxic lesions of serotonin (5-HT)-containing neurons significantly deplete the SP innervation of the ventral horn (Johansson et al., 1981). *In vitro* electrophysiological studies of neonatal spinal cord revealed a profound excitatory effect of SP on spinal motoneurons that can be blocked by SP antagonists (Yanagisawa and Otsuka, 1990). Interestingly, intrathecal injection of some SP antagonists is neurotoxic to motoneurons, suggesting that SP may also exert trophic influences on these cells (Post and Paulsson, 1985).

The intermediolateral cell column (IML) of the thoracic and rostral lumbar spinal cord is also a major target of the descending medullary SP projection (Helke, et al., 1982; Johansson, et al., 1981; Menétrey and Basbaum, 1987; Reddy, et al., 1990). Direct projections to sympathetic preganglionic neurons have been demonstrated and electrolytic lesions of the ventral medulla reduce the SP content of the IML. Iontophoresis of SP evokes a moderate increase in the firing rate of sympathetic preganglionic neurons (Backman et al., 1990) and behavioral studies demonstrated that the increased heart rate and blood pressure produced by electrical stimulation of the ventral medulla can be blocked by intrathecal administration of a SP antagonist (Loewy and Sawyer, 1982). Taken together these data indicate that both motoneuronal and cardiovascular controls exerted from the rostral ventral medulla are mediated

in part by SP-containing bulbospinal axons.

The fact that selective receptor antagonists block the action of SP indicates that a specific receptor mediates these effects, a conclusion supported by radioligand binding studies. Pharmacological studies have, in fact, established that SP acts preferentially at the NK-1 subtype of tachykinin receptor. Neurokinin A (NKA), a peptide that co-occurs in primary afferent terminals with SP, binds preferentially to the neurokinin-2 (NK-2) receptor. Neurokinin B is the presumed endogenous ligand for the neurokinin-3 (NK-3) receptor. Since the effects of substance P are blocked by NK-1 antagonists, we will refer to this receptor as the substance P receptor (SPR).

Consistent with the diverse functional effects of SP in the spinal cord, both *in situ* hybridization studies of the distribution of the mRNA encoding the SP receptor and receptor binding analysis with radiolabelled SP demonstrated that there is a widespread distribution of the NK-1 receptor, in dorsal horn, IML and ventral horn of the spinal cord. Neither of these techniques, however, provides the resolution necessary to identify the populations of neurons that express the receptor. *In situ* hybridization labels cell bodies (provided the message is sufficiently abundant), but the absence of dendritic labelling makes it impossible to identify the morphology of labelled neurons (Kiyama et al., 1993). Radioligand binding studies label both cell bodies and dendrites, but the resolution is not adequate to identify individual neurons (Charlton and Helke, 1985; Helke et al., 1986; Yashpal et al., 1990).

In a preliminary study, we reported results obtained with an antibody directed against the carboxyl terminal residue of the rat substance P receptor (Liu et al., 1994). We found that the SPR completely decorates the somatic and dendritic surface of subpopulations of neurons throughout the brain and spinal cord. In the present study, we provide a detailed light microscopic analysis of the distribution of substance P receptor-immunoreactive neurons at all levels of the rat spinal cord.

MATERIALS AND METHODS

Characterization of the SPR antibody:

The anti-SPR antibody was raised against a 15 amino acid peptide sequence (SPR393-407) at the carboxyl-terminus of the rat substance P receptor (Vigna et al., 1994). Using glutaraldehyde, the synthetic peptide was conjugated to bovine thyroglobuline for immunization. The antiserum used in this study (#11884-5) recognized a protein band of 80-90kD on Western blots of membranes prepared from cells transfected with the rat SPR. The cells could also be immunostained with the antiserum and the staining was blocked by preabsorbing the antiserum with SPR393-407. These observations taken together with the fact that there is a very high correspondence of the distribution of SPR immunoreactivity and ^{125}I-SP binding in the brain strongly suggest that the the antibody is selectively localizing the SP receptor. However, since we cannot completely rule out possible cross-reactivity of the antiserum with other proteins, we refer to the staining as SPR immunoreactivity.

Immunocytochemistry:

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at UCSF. Experiments were performed on male Sprague-Dawley rats (260-300g; Bantin and Kingman, Fremont, CA). The rats were deeply anesthetized with ketamine (60 mg/kg) and xylazine (6.0 mg/kg) and then perfused intracardially with 50 ml of 0.5M phosphate-buffered saline (PBS) followed by 500 mL of 2% paraformaldehyde, 0.5% glutaraldehyde fixative in an 0.1M phosphate buffer (PB). After perfusion, the brain and spinal cord were removed, postfixed for five hours in the same fixative and then cryoprotected in 30% sucrose in 0.1M PB. Immunostaining according to the avidin-biotin peroxidase method of (Hsu et al., 1981) using commercially available kits (Vectastain, Burlingame, CA) was performed on 50 μm spinal cord sections cut in either the coronal or sagittal planes on a freezing microtome. The primary antiserum was routinely diluted to 1:50,000 and Triton X-100 was used in all incubation steps. To localize the HRP immunoreaction product we used a nickel-intensified diaminobenzidine protocol with glucose oxidase. Reacted sections were mounted on

gelatin-coated slides and dried overnight. The following day, the sections were dehydrated and coverslipped with Cytoseal.

Preincubation of the primary antiserum with 10 μg/ml of SPR393-407 abolished the staining. The distribution of immunoreactivity was analyzed according to the cytoarchitectonic descriptions of (Molander et al., 1984; Molander et al., 1989) as a guide to laminar organization of the different levels of the spinal cord. We measured cell body diameters directly in the microscope; no corrections were made. Finally we compared the pattern of SPR immunoreactivity with that revealed in autoradiograms of ^{125}I-SP binding according to procedures previously described (Mantyh et al., 1989).

To determine whether the SPR is located on primary afferent fibers, in addition to immunostaining dorsal root and trigeminal ganglia, we examined tissue from rats that underwent unilateral L4-S1 dorsal rhizotomy (n=3) or unilateral sciatic nerve ligation (n=3). For the dorsal rhizotomies, the rats were anesthetized with ketamine and xylazine and then we performed a laminectomy of the appropriate lumbar vertebra. The dura was incised, the L4-S1 dorsal roots were identifed and then cut on the right side. Next the exposed spinal cord was covered with Gelfilm and the overlying muscle and skin was sutured. After a one week survival period, the rats were reanesthetized and perfused. Spinal cord tissue from these animals was processed for immunocytochemistry as described above. For sciatic nerve ligation, the rats were anesthetized with ketamine and xylazine so that the sciatic nerve could be exposed in the midthigh. Next a tight ligature was tied around the nerve; this served to block axoplasmic transport from the dorsal root ganglion (DRG) to the periphery so that we could examine the nerve for build-up of SPR immunoreactivity, proximal to the ligature. The rats survived one week. After fixation and cryoprotection of the tissue, the sciatic nerve and ligature were cut at 10 μm on a cryostat and the tissue immunoreacted as described above.

To selectively eliminate small diameter primary afferent fibers, we also studied three rats that were treated with neonatal capsaicin (100 mg/kg; sc) one day after birth. This procedure produces a more complete destruction of unmyelinated primary afferents than does

the more traditional 50mg/kg injection on postnatal day 2. When the rats weighed 260-300 grams, they were anesthetized and perfused for immunocytochemistry. The spinal cord tissue was processed as above.

RESULTS:

General observations on SPR immunoreactivity in the spinal cord:

The overall pattern of labelling is comparable to that observed in radiolabelled ligand binding studies. SPR immunoreactivity is concentrated in superficial dorsal horn, and along its medial border (Figures 1,2). As we reported previously (Liu, et al., 1994), the predominant pattern of labelling consisted of SPR immunoreactivity that decorated most of the somatic and dendritic surface of subpopulations of neurons in the spinal cord (Figures 3,4). Since the extensive labelling of the dendritic tree resulted in "Golgi like" staining of SPR-laden neurons we were able to identify subpopulations of immunoreactive neurons and to compare cell morphology with that revealed in Golgi studies and in studies that electrophysiologically characterized and intracellularly labelled single dorsal horn neurons.

In addition to the discrete labelling pattern of individual neurons, we detected staining in some regions of the brain and spinal cord that was of a much more diffuse, non-cellular quality. The staining quality was at times particulate, but more often it appeared as a cloud of immunoreactivity, with no underlying structure discernible, i.e. we could not determine whether the SPR was located on axonal or dendritic structures. In some cases, notably in the ventral horn, we could recognize clusters of motorneurons within a cloud of SPR immunoreactivity (Figures 1F, 5B,C, 6F, 8D). Nowhere in the spinal cord did we find evidence that the SPR is expressed on axons.

In this study we characterized the staining pattern and identified subpopulations of SPR-immunoreactive neurons at all segmental levels of the cord. Although the pattern of SPR immunoreactivity was comparable at the different levels, we recorded significant segmental differences, with respect to both the numbers and density of cell body labelling (Figure 1). In general, lamina I contained the highest density of SPR, throughout the entire rostrocaudal extent

of the spinal cord. Neurons surrounding the central canal, within and adjacent to lamina X, also expressed SPR at most levels of the cord. A great surprise was the paucity of labelled neurons in the substantia gelatinosa, at all segmental levels. In contrast to the relatively consistent pattern of SPR immunoreactivity in the superficial dorsal horn, the pattern of staining in the deeper dorsal horn and ventral horn varied at different segmental levels. Since the most intense staining for the SPR was found in the lumbar spinal cord, we will begin our analysis in this region; similarities and differences at other levels will then be described.

Lumbar Cord

The marginal layer (lamina I)

Figure 1 illustrates the overall pattern of SPR immunoreactivity in the spinal cord and demonstrates that the densest labelling is in lamina I, which contains many heavily labelled SPR-immunoreactive neurons. Since most of the staining was located on dendritic processes that arborized rostrocaudally in the marginal zone, these neurons were best viewed in sagittal section. The most common type of SPR-immunoreactive neuron in lamina I had a fusiform cell body that measured between 30 to 40 μm in the longest dimension, and an extensive rostrocaudal dendritic tree, which extended up to 400 μm from either pole of the cell body (Figure 2E). The SPR-immunoreactive processes of marginal neurons typically remained confined to lamina I and the outer portion of lamina II; a few SPR-immunoreactive marginal neurons had processes that reached into the deeper portion of lamina II and lamina III (Figure 2D). Most of the SPR-immunoreactive dendrites in the marginal layer were aspiny, and as such correspond to the fusiform neuron in the Lima and Coimbra (1989) classification. A much larger, albeit less common SPR-immunoreactive neuron, with a fusiform cell body, was seen in transverse section (Figure 2B). The dendrites of these neurons arborized along the curvature of the marginal zone, similar to the Waldeyer neurons. Occasionally, we observed labelled cell bodies in the white matter dorsal to lamina I.

To obtain an estimate of the percentage of neurons that expressed the SPR in lamina I

we counterstained some sections with Cresyl Violet and counted double-labelled neurons. We estimate that approximately 5 percent of the neurons in lamina I were SPR-immunoreactive. This is only a rough estimate, because of the difficulty distinguishing glial cells from small neurons; however, it underscores the fact that a remarkably small number of neurons in lamina I expresses the receptor. This indicates that despite the intense labelling in lamina I, most of the labelling is associated with the dendritic arbors of a small number of neurons.

The substantia gelatinosa (lamina II)

The substantia gelatinosa (SG) contrasts sharply with lamina I, in that it contains much lower amounts of SPR immunoreactivity. In fact, we recorded very few SPR-labelled neurons with somata located within the SG. Importantly, the population of labelled neurons in the SG did not include any that resembled the stalk or islet neurons, which best characterize the interneuron population of lamina II. The few labelled neurons of the SG were large, multipolar neurons, approximately 40 μm in diameter, with an elaborate and extensive dendritic arbor that extended up to 400 μm in the rostrocaudal direction. The dendritic arbor was characteristically "V", or fan shaped, with many of the dendrites reaching dorsally into the marginal layer (Figures 2F,G). Neurons with somewhat similar morphology were also commonly located in lamina III (vide infra).

Despite the paucity of SPR-immunoreactive cell bodies, the SG contains a significant population of large dendrites that derive from areas outside of the SG, a few from lamina I (vide supra) and many more from neurons located in lamina III. In the lumbar enlargement, these dendrites were concentrated in the medial half of the SG and arose from neurons located in the medial half of the nucleus proprius. Figure 2A illustrates the contrast between the medial half of the SG, which contains large numbers of labelled dendrites, and the lateral half, which is largely devoid of SPR-immunoreactive processes.

The nucleus proprius and the neck of the dorsal horn

The nucleus proprius of the dorsal horn, including laminae III and IV, and the neck of the dorsal horn (laminae V and VI) contained a heterogeneous population of SPR-

immunoreactive neurons. On the basis of dendritic arborization, we have classified these neurons into four groups. The most notable feature of the first group of labelled neurons is a large primary dendrite (measuring up to 300 microns in length) that coursed dorsally through the SG, often into the marginal layer. The primary dendrite appeared to have few secondary branches; however, because of the extensive dendritic labelling of SPR-immunoreactive marginal neurons, we could not rule out the possibility that these neurons branched in lamina I. The cell body of neurons in the first group was large 30 μm and pyramidal and was most often found in lamina III, somewhat less commonly in lamina IV (Figure 3C). These neurons are morphologically similar to the antenna cells described in Golgi preparations (Maxwell et al., 1983; Rethelyi and Szentagothai, 1973).

The second group of SPR-immunoreactive neurons was distributed in laminae III through V. These neurons (cell body ~30 μm in diameter) were multipolar, with thick dendrites that arborized in all directions and in all planes. The mediolateral dendritic spread reached up to 400 μm. The dorsoventral spread was also large, often traversing several laminae. Neurons with similar morphology could be recognized in sagittal section, a view that established that some of the dorsally-directed dendrites also spanned up to 400 μm in the rostral caudal direction. Like antenna cells, the dendrites of neurons of group two, with cell bodies located in lamina III, also often extended dorsally into the marginal layer (Figures 3A,B). In contrast, the dendrites of neurons located more ventrally, in lamina V, rarely penetrated the SG. The latter neurons were particularly common in the sacral spinal cord (Figures 4A,B).

The third group of neurons was concentrated along the medial border of laminae IV-V (Figures 8A,B). These neurons had small, fusiform cell bodies (20-30 μm in diameter), with dendrites that arborized dorsally and ventrally; the total dorsoventral dendritic length ranged from 300 to 500 μm. The dendrites were thin, aspiny, generally unbranched, and did not extend in the mediolateral plane. In several instances thin dendritic processes of these neurons penetrated the medially adjacent dorsal column white matter (Figures 4C-E). The fourth group of labelled neuron was scattered throughout laminae III to VI. These neurons had small, round

cell bodies, with short and relatively fine dendrites that arborized in the rostrocaudal plane. Dendrites of these neurons never arborized in the superficial dorsal horn (laminae I and II; Figure 3D).

Ventral horn

At all levels of the lumbar spinal cord, we recorded a very light, diffuse pattern of SPR immunoreactivity in the ventral horn. The immunoreactivity appeared as a veil surrounding a cluster of motoneurons. Rarely were individual neurons labelled as intensely, or as distinctly as in the dorsal horn. Two motoneuron groups in lamina IX, the latero-medial nucleus (LMN) and the ventro-medial nucleus (VMN), that probably contain levator ani motoneurons (Molander, et al., 1984) were diffusely outlined, in a very light cloud of SPR immunoreactivity. At more rostral levels of the lumbar cord, we recorded clusters of large motoneurons in laminae VII and VIII that were lightly stained for SPR. Often the latter had extensive dorsally-directed dendritic bundles that reached into the intermediolateral cell column and the neck of the dorsal horn (Figures 5B,C). In some cases, the SPR-immunoreactive dendrites extended well into the ventral and ventrolateral white matter, occasionally contacting the dendritic bundles that arose from neurons located in the contralateral laminae VII, VIII and IX. In general the dendritic labelling was smooth, however, some of the labeled processes in the ventral horn were beaded. Although these structures were more axonal in morphology, we often could trace the beaded processes back to a parent dendrite.

The intermediolateral column

In the more rostral sections of the lumbar cord (L1-L3), we recorded very densely stained neurons in the region of the intermediolateral column (IML; Figure 5A). A detailed description of the labelling in this region is provided in the section on the thoracic spinal cord.

The lateral spinal nucleus

At all levels of the spinal cord we recorded SPR-immunoreactive neurons in the lateral spinal nucleus (LSN) located in the dorsalmost part of the dorsolateral white matter. Most of the neurons in the LSN were small, with dendrites that radiated in all directions from the cell

body (Figure 5A).

The central canal

In transverse sections of the more rostral segments of the lumbar cord (L1-L3), we observed a dense concentration of SPR-immunoreactive processes surrounding the central canal (Figure 1C). Only rarely could we identify labelled cell bodies. In the saggital plane, however, the cellular layout of the SPR neurons was apparent. Many small round cell bodies that were densely stained for SPR surrounded the central canal. We also recorded thin, beaded SPR-immunoreactive processes that ran parallel to the central canal, occasionally penetrating through the ependymal lining of the canal and ending freely within the canal (not shown). Comparable beaded processes that extended dorsally and ventrolaterally from the central canal could also be seen in transverse sections.

Cervical Spinal Cord

The overall pattern of SPR expression in the cervical spinal cord resembled that observed in the lumbar cord, however, the SPR immunoreactivity in the cervical spinal cord was considerably less abundant (Figure 1A). This was the case even when tissue from cervical and lumbar spinal cord from the same rat was immunoreacted simultaneously.

The superficial dorsal horn: laminae I and II

As in the lumbar cord, lamina I contained fusiform neurons with dendrites that had an extensive rostrocaudal spread. The overall density of staining, however, was generally lower than in the lumbar cord (Figure 6C). The substantia gelatinosa contained almost no SPR immunoreactivity. The small amount of staining that was observed was found predominantly on processes that travelled through the SG; these processes derived from marginal neurons with ventrally directed dendrites or from the dorsally directed dendrites of dorsal horn neurons in the nucleus proprius. As in the lumbar cord, although less striking, the labelling of dorsally directed dendrites in the SG was concentrated in its medial half.

The deeper dorsal horn: laminae III-VI

The deeper dorsal horn of the cervical spinal cord contained a sparse population of SPR-immunoreactive neurons. The most common type of labelled neuron was a bipolar neuron with dorsally and ventrally directed dendritic branches. These neurons were best viewed in the transverse plane; their cell bodies were typically located in the medial portion of laminae IV or V, and their dendrites followed the medial edge of the spinal gray matter. These neurons had large, often fusiform cell bodies, with dendrites that extended into the laterally adjacent white matter. In contrast to the lumbar cord, we rarely found in lamina III, the very large multipolar neuron with dorsally-directed dendrites that reached the marginal layer (Figures 6A,G,I).

Ventral horn

In addition to the overall light staining that characterized the ventral horn of the lumbar cord, we found very pronounced staining around certain clusters of motoneurons. As in the lumbar cord, it was difficult to identify individual neurons within the cloud of diffuse reaction product that surrounded the cell bodies. In sagittal sections taken through mid cervical segments (C3/4-C5/6), we observed groups of motoneurons that were lightly stained. These neurons had extensive dendritic branches that branched both dorsally and ventrally (Figure 6F).

Thoracic Spinal Cord

The dorsal horn

Figure 7A illustrates that SPR immunoreactivity is densely distributed in lamina I of the thoracic cord. The morphology of the labelled neurons resembled the lamina I neurons described for the lumbar cord. The long rostrocaudal spread of the labelled dendrites was strictly confined to lamina I. In fact, the remainder of the dorsal horn (laminae III-V) was almost devoid of staining. The large lamina III neurons with dorsally directed dendrites that characterized the lumbar cord were rarely found in the thoracic cord. In contrast to the cervical and lumbar cord, we did not record SPR staining in the ventral horn of the thoracic cord.

The intermediolateral cell column (IML)

The most striking labelling in the thoracic cord was recorded in the IML. Transverse sections revealed that many of these neurons in the IML had dendrites that extended medially, to the area around the central canal (Figure 7C). Since the dendrites of several closely packed IML neurons formed tightly packed bundles that traversed the grey matter, it was often difficult to resolve single dendritic processes. By contrast, we could readily identify laterally directed dendrites of IML neurons that arborized widely in the lateral white matter. Sagittal sections revealed that the SPR-immunoreactive IML neurons were not continuously arrayed. Rather they were grouped into clusters, separated by regions that contained densely packed dendritic arbors of the IML neurons, but no labelled cell bodies (Figure 7B). The presence of a marked dorsoventral arborization of some of the dendritic trees of IML could also be appreciated in sagittal section. Many dendrites of IML arborized in the dorsolateral funiculus, extending into the region of the lateral spinal nucleus.

The area around the central canal (lamina X) and the intercalated nucleus

In transverse sections of the thoracic cord, we observed dense punctate SPR immunoreaction product in the neuropil surrounding the central canal, lamina X (Figure 7C). In sagittal section, we noted that the dendrites of these neurons course rostrocaudally, which suggests that the dense punctate staining observed in transverse section consisted of labelled bundles of dendrites cut in cross section. In the transverse plane, we occasionally observed that some of the neurons around the central canal had dendrites that coursed laterally, towards the IML. These dendrites were often interdigitated with the medially directed dendritic bundles those arose from neurons in the IML.

The intercalated nucleus, located midway between the central canal and the IML, contains relatively lightly stained SPR-immunoreactive neurons with somata that measured 20 μm in diameter (Figure 7C). Dendrites of SPR-immunoreactive neurons in the intercalated nucleus extended both medially and laterally, to the area around the central canal and the IML, respectively.

Sacral Spinal Cord

The dorsal horn

The morphology of marginal neurons in the sacral cord that expressed SPR immunoreactivity (Figure 8A) was comparable to that of the lumbar and cervical levels. In contrast to these other levels, however, we recorded more marginal neurons with dendrites that penetrated ventrally. Many of the dendrites reached the neck of the dorsal horn, in the region of lamina V. As at all other segments of the spinal cord, the SG contained few labelled neurons, the exception being isolated, densely stained, very large multipolar neurons. The majority of the labelled processes in the SG of the sacral cord derived from dendrites of large neurons in laminae III and IV; the latter were very similar, although more common than those observed in the lumbar cord. Figure 8B illustrates that these sacral neurons have very large cell bodies (up to 40 μm in diameter), and a very extensive, large caliber dendritic tree that arborizes dorsally, medially and laterally. Some of the daughter branches of these neurons reached the marginal layer. In some cases, the medially directed dendritic branches crossed the midline, extending well into the contralateral gray matter (Figure 8B). As in more rostral segments, we also recorded labelling around the central canal. In the sacral cord, it was somewhat particulate and rather diffuse.

Ventral horn and parasympathetic preganglionic neurons

We also recorded very prominent labelling in the ventral horn of the sacral cord in Onuf's nucleus, which contains motoneurons that innervate somatic muscles of the bladder. Two types of immunoreactivity were recorded (Figure 8D). A diffuse cloud of immunoreactivity filled the confines of the nucleus; in the central region of the nucleus, a darker pattern of staining outlined individual motoneurons. In addition to this somatic motoneuron pool of the sacral cord, we also observed that many neurons in the region of the parasympathetic preganglionic nuclei of the sacral cord were immunostained for the SPR (Figure 8C). In transverse section, we noted that these neurons typically had relatively large

cell bodies with extensive dendritic branches that arborized medially, to the central canal, and laterally, into the lateral white matter. Although they were more lightly stained, these neurons were comparable to those observed in the IML of the thoracic cord.

Primary Afferents

We did not find expression of the SPR in neurons of the dorsal root or trigeminal ganglia. Since the protein may be synthesized by DRG neurons, but not stored in the cell bodies, we performed three additional experiments designed to enhance detectability of the protein. None of these experiments provided evidence that the SPR is present in primary afferents. Specifically, we found no consistent change in the level of SPR immunoreactivity in the dorsal horn ipsilateral to a multiple dorsal rhizotomy. We also did not detect damming of SPR immunoreactivity proximal to the sciatic nerve ligation, which would have been expected if the receptor were transported by primary afferents from the DRG. That result also indicates that ventral horn motoneurons, which appear to express the receptor, do not transport it distally in motor axons, at least in immunocytochemically detectable levels. Finally, neonatal capsaicin treatment, which selectively destroys unmyelinated afferents, did not produce a notable decrease in the amount of SPR immunoreactivity in the superficial laminae of the dorsal horn, compared to normal rats. Although we did not examine the peripheral nerve for numbers of unmyelinated axons, the high dose used (100 mg/kg) consistently results in a significantly loss (Nagy et al., 1983).

Trigeminal nucleus caudalis

Since the trigeminal nucleus caudalis (TNC) of the spinal trigeminal nucleus is homologous with the spinal cord dorsal horn, we also examined this region for SPR, to determine if comparable patterns of staining are found. As in the spinal cord, lamina I of the TNC contained many heavily labelled SPR-immunoreactive neuron; lamina II, the substantia gelatinosa, was noticeably devoid of SPR-immunoreactive neurons (Figure 9A). A distinct

band of labelling was located in the most dorsal part of the nucleus magnocellularis, which corresponds to lamina III of the spinal cord dorsal horn (Figures 9B,C). Many of the labelled neurons in this region were multipolar; several had dorsally directed dendrites that traversed the overlying SG to reach the marginal layer (Figure 9B-D).

DISCUSSION

The present study demonstrates that the SP receptor is expressed by morphologically distinct populations of neurons in the rat spinal cord and in homologous regions of the trigeminal nucleus caudalis. Taken together with the already established functional properties of neurons in regions that express the receptor, and with the well documented distribution of SP containing terminals, the present results permit a more precise characterization of the morphology and properties of spinal cord neurons that express the SPR and thus are likely to respond to SP. These results make it possible to better detail the circuits through which substance P-containing primary afferent influence the firing of second order nociresponsive neurons. We recognize the possibility that high concentrations of SP may exert effects via the NK-2 or NK-3 receptors, which are also expressed in the dorsal horn, however, since the NK-1 receptor binds SP with a hundredfold higher affinity than does the NK-2 or NK-3 receptors, an action at the NK-1 receptor probably predominates.

Primary afferent fibers do not express the Substance P receptor

Although previous studies provided no direct evidence that primary afferent fibers express the SP receptor, the possibility was suggested from studies of Randic and colleagues (Randic, et al., 1990; Rusin, et al., 1992), which demonstrated that the release of glutamate can be regulated by peptides derived from primary afferents, namely SP and calcitonin gene-related peptide (CGRP). Our results, however, are in complete agreement with *in situ* hybridization and ligand binding studies (Helke, et al., 1986; Kiyama, et al., 1993; Maeno et al., 1993; Mantyh, et al., 1989; Sivam and Krause, 1992). We found no staining of DRG neurons, even after manipulations designed to facilitate detection of the receptor in afferent fibers. Our results are also consistent with studies that found no change in central or peripheral binding of SP after ricin treatment (Buck et al., 1986; Helke et al., 1990) and with others that found an increase in dorsal horn labelling after dorsal rhizotomy (Aanonsen and Seybold, 1991; Yashpal et al., 1991), rather than a decrease, which would have been expected if the receptor were located on the primary afferents. These results indicate that primary afferents do not express a SP

autoreceptor that is targetted by SP. Although we cannot comment on the presence or absence of a primary afferent CGRP receptor, the possibility must be considered that the modulation of glutamate release by SP and CGRP is secondary to activation of interneurons that, in turn, presynaptically regulate the primary afferent.

The absence of a SPR on primary afferents is also relevant to the mechanisms through which SP contributes to peripheral neurogenic inflammatory mechanisms (Devor et al., 1989; Holzer, 1988; Lembeck et al., 1982; Xu et al., 1992). Specifically, there is evidence that plasma extravasation from postcapillary venules involves SP release from the peripheral terminals of primary afferents. Our results suggest that any contribution of SP to this inflammatory process is not via a direct action on the primary afferent terminal.

Laminar distribution of SPR-immunoreactive neurons

We distinguished two populations of lamina I neurons that express the SP receptor. The cell type that predominated had a fusiform cell body and long dendrites that coursed rostrocaudally. The morphology of these neurons is similar to the fusiform class of spinoparabrachial projection neurons, which responds to and transmits nociceptive information, via the parabrachial area to the amygdala (Hylden et al., 1985; Hylden et al., 1986; Light et al., 1993; Lima and Coimbra, 1989; Menétrey et al., 1982). Some of the SPR-immunoreactive marginal neurons also had dendrites that arborized ventrally, through the substantia gelatinosa. In Golgi studies, Beal (1979) described subpopulations of marginal neurons with comparable dendritic arbors. The second type of marginal SPR-immunoreactive neuron resembled the large Waldeyer neuron and was much less common. This neuron was best recorded in transverse section, which revealed the mediolateral arborization of its dendrites, along the margin of the dorsal horn. In preliminary studies we have double-labelled SPR immunoreactive neurons after retrograde tracer injections in different supraspinal targets, including the parabrachial area and the thalamus. These results suggest that the marginal layer contains a population of SP responsive projection neurons, a conclusion that complements studies that demonstrated SP inputs to projection neurons in lamina I (Priestley et al., 1982).

Based on the dense staining of SP in the marginal layer, one might have predicted that a large percentage of neurons in lamina I are contacted by SP-containing primary afferent terminals. We cannot rule out this possibility, however, our results indicate that only a subpopulation of neurons in the marginal layer express the receptor. In fact, a comparison of the numbers of labelled cell bodies with the number of neurons identified in Nissl preparations revealed that the percentage of neurons that express the SP receptor in lamina I is remarkably small, about five percent. This conclusion is supported by our recent studies of neurons that coexpress the SPR and the c-fos proto-oncogene in response to noxious stimulation; the vast majority of fos-immunoreactive neurons in the marginal layer were *not* double labelled (unpublished observations). The paucity of SPR-immunoreactive neurons, taken together with the their differential distribution at different rostrocaudal levels of the cord suggests not only that most nociresponsive neurons in the marginal layer are not directly driven by SP, but also that there may be a greater contribution of SP to the transmission of nociceptive messages to lumbosacral than to cervical dorsal horn lamina I neurons. Furthermore, since the cervical spinal cord of the rat contains a significantly greater number of lamina I neurons at the origin of the spinothalamic tract (STT) (Granum, 1986) our results suggest that SP makes only a minor contribution to the transmission of nociceptive messages by STT neurons in the marginal layer of the rat. This assumes, of course, that under physiological conditions, SP is not a ligand for other tachykinin receptors, specifically, the NK-2 receptor.

Lamina II: the substantia gelatinosa

In light of the dense innervation of SP terminals in the SG, particularly its outer aspect, the paucity of SPR-immunoreactive neurons in the SG was unexpected. This result, however, agrees with electrophysiological studies that found that only a small percentage of SG neurons are excited by SP iontophoresis (Duggan et al., 1979). Furthermore, recent studies using an *in vitro* preparation of the spinal cord concluded that the central effects produced by capsaicin, which is presumed to selectively stimulate small diameter primary afferents, are mediated by the NK-2, not the NK-1 receptor (Nagy et al., 1993). On the basis of these results the authors

hypothesized that NKA, rather than SP, mediates the primary afferent evoked responses in SG interneurons. Similar conclusions were drawn from studies of inflammation associated induction of dynorphin in superficial dorsal horn (Fleetwood-Walker et al., 1990; Fleetwood-Walker et al., 1993; Munro et al., 1993). On the other hand, other studies indicate that although the central effects of C-fiber stimulation in the normal rat are NK-2 mediated, the C-fiber mediated hyperalgesia observed under injury conditions has an NK-1 sensitive component (Thompson et al., 1994).

It is significant that we did not observe SG neurons that resembled the stalked or islet cell interneuron, the two most common SG interneurons that have been identified in Golgi and intracellular electrophysiological studies (Bennett et al., 1980; Gobel, 1975; Gobel, 1978). Since stalked cell axons arborize in lamina I, it has been proposed that the stalked cell is an interneuron that transmits nociceptive information from small diameter primary afferents to the marginal projection neurons. Electrophysiological studies of paired recordings from SG neurons and overlying marginal neurons provided support for this hypothesis (Price et al., 1979). Our results indicate that it is unlikely that information transfer from SG neurons to marginal projection neurons is initiated by activity in SP-containing primary afferent inputs. The few labelled neurons that we observed were large and multipolar. These neurons resemble the large central cell described by Beal (Beal, 1983), which is presumed to correspond to the very limited population of projection neurons located in the SG (Giesler et al., 1978). Alternatively, some of these neurons may be displaced members of the large SPR-immunoreactive neurons in lamina III.

The profound mismatch between the dense concentration of SP terminals and the paucity of SPR in the SG raises the question as to the target of the SP terminals. One possibility is that the dorsally directed dendrites of the SPR-immunoreactive lamina III neurons are targetted by SP released in the SG. On the other hand, there is considerable evidence that peptides can diffuse considerable distances from their site of release (vide infra). It is, therefore, possible that the SP released dorsally acts upon SPR-immunoreactive neurons located

in the neck of the dorsal horn. This observation is consistent with the fact that the SP receptor is distributed over almost the entire somatic and dendritic surface of labelled neurons and that most of the labelled membrane is *not* apposed by SP immunoreactive terminals (Liu et al., 1994). To what extent the SPR located on deep dorsal horn neurons is acted upon by SP that diffuses from more superficial laminae and under what conditions this occurs is not clear. Conceivably, it arises under conditions of persistent noxious stimulation associated with inflammation and maintained nociceptive inputs into the spinal cord. This, of course, is not the first example of SP/SPR mismatch. In the brain, the highest concentration of SP is found in the substantia nigra, a region where SP receptors are not at all detectable (Shults et al, 1984; Mantyh et al, 1989).

The nucleus proprius (laminae III and IV)

The population of large, multipolar SPR-immunoreactive neurona in lamina III was particularly striking. These neurons had several large dendrites that arborized dorsally, in the SG. In some cases the dendritic arbor of a single neuron spanned the mediolateral extent of the dorsal horn and in sagittal section we noted that some SPR-immunoreactive neurons in lamina III had dendrites that extended rostrocaudally over 400 μm. The morphology of these cells is very similar to the antenna cell described in Golgi studies (Maxwell et al., 1983; Rethelyi and Szentagothai, 1973)

Interestingly, although the majority of neurons in lamina III and IV receive large diameter primary afferent input and respond to non-noxious stimulation, nociceptive neurons with similar morphology have been identified in intracellular electrophysiological studies in the cat (Maxwell et al., 1983). Antidromic activation from the cervical cord established that these were spinocervical tract neurons. Presumably the nociceptive input derives from small diameter afferents that contact the distal dendrites of these neurons in the superficial dorsal horn. Electron microscopic analysis of the distal dendrites of antenna cells supports this hypothesis. Since there is a high concentration of SP in laminae I and in outer II, but very little in lamina III, it follows that these neurons receive their SP input from primary afferents that terminate in

laminae I and II.

Since we found few immunoreactive cell bodies in the SG and because many fewer dendrites of SPR-immunoreactive marginal neurons penetrated the SG, it is likely that the dorsally directed lamina III cell dendrite is the predominant target of SP terminals in the SG. An interesting feature of the SPR-immunoreactive dendritic arbors in the SG is that they were not found uniformly. They were most numerous in the lumbar and cervical enlargements, particularly in the medial half of the SG, and in the sacral spinal cord. Although it is our impression that the dendrites in the medial part of the SG arose predominantly from SPR-immunoreactive neurons in lamina III, marginal neurons with ventrally arborizing dendrites are, in fact, concentrated in the medial half of lamina I (Beal, 1979). It is also of interest that there is a greater density of SP immunoreactivity in the medial than in the lateral portion of lamina II (Hammond and Ruda, 1991).

Since there is a distinct topographic organization of cutaneous afferent inputs to the dorsal horn, these results raise the possibility that the SP regulation of lamina III neurons whose dendrites arborize in the SG is more associated with inputs from the glabrous skin (Shortland et al., 1989). Although previous studies emphasized the distinction in SP content of muscle and cutaneous afferents (McMahon et al., 1984), our results indicate that there is also a difference in the glabrous and hairy skin peptidergic afferent input to the dorsal horn.

The neck of the dorsal horn:

Since iontophoresis of SP excites nociresponsive neurons in laminae V-VII (Henry, 1976; Radhakrishnan and Henry, 1991; Salter and Henry, 1991), the presence of SPR-immunoreactive neurons in these regions was expected. Precisely how these neurons receive a nociceptive SP input is however, not clear. Although scattered SP terminals are located in the neck of the dorsal horn (Barber et al., 1979; Hökfelt et al., 1975; Ljungdahl et al., 1978), the highest concentration is clearly located in lamina I and in outer lamina II. Furthermore, there is little evidence for a direct C fiber input to the neck of the dorsal horn; thus any monosynaptic C fiber input (possibly SP-mediated) to these neurons must be on their dorsally-located dendrites.

Although some of the SPR-immunoreactive neurons in the region of laminae V had dendrites that extended into the SG, in the majority of cases the dendrites ended ventral to the SG. Thus a primary afferent derived SP input to nociresponsive SPR immunoreactive neurons in the neck of the dorsal horn is even less likely. Although De Koninck et al (1992) demonstrated that some nociresponsive neurons in the region of lamina V are contacted by SP-immunoreactive terminals, the paucity of SP terminals in the nucleus proprius and in the neck of the dorsal horn suggests that these contacts are atypical. It is also unclear what percentage of nociresponsive neurons in the neck of the dorsal horn responds to iontophoresis of SP.

The other possibility, as described above, is that SP can diffuse from its site of release in the superficial dorsal horn, to act upon more ventrally located neurons that express its receptor. There is, in fact, considerable evidence that SP can diffuse a significant distance within the spinal cord parenchyma. First, high intensity stimulation evokes the release of SP into the spinal CSF (Go and Yaksh, 1987); this presumably derives from release in the superficial laminae. Conversely, intrathecal injection of SP evokes a profound caudally directed biting and scratching behavior (Hylden and Wilcox, 1981; Larson, 1988; Rackham et al., 1981); this must result from diffusion of the peptide from the CSF into the spinal cord tissue. Using antibody coated microelectrodes, Duggan and colleagues and others (Duggan et al., 1990; Duggan et al., 1992; Hope and Lang, 1990; Hylden and Wilcox, 1981; Larson, 1988; Rackham et al., 1981; Schaible et al., 1990) have characterized the spread of a variety of peptides in the dorsal horn. In the presence of CGRP and other inhibitors of peptide degradation, the authors found considerable diffusion of SP from the superficial laminae. The fact that the SP receptor is expressed over almost the entire surface of the SPR-positive neuron, of course, increases the likelihood that SP would reach and activate them.

Sympathetic preganglionic neurons (SPN):

Consistent with NK-1 binding studies (Buck et al., 1986; Charlton and Helke, 1985; Helke et al., 1986; Shults et al., 1984; Yashpal et al., 1990), we found SPR-immunoreactive neurons in each of the three major regions of the rat spinal cord that contain sympathetic

preganglionic neurons (SPN), the intermediolateral cell column, the intercalated nucleus and the central autonomic nucleus. The immunohistochemical analysis revealed that many of the SPR-immunoreactive neurons had dendrites that coursed mediolaterally from the IML to the central canal, which is consistent with a morphological analysis of antidromically activated SPNs in the IML (Forehand, 1990). Heavily labelled dendrites in the dorsolateral funiculus also arose from the labelled neurons in the IML. There is, in fact, considerable evidence that substance P contributes to the regulation of sympathetic function. Ionotophoresis of SP excites preganglionic neurons in the IML (Backman et al., 1990) and intrathecal injection SP in the region of the IML at the T2 or T9 segments respectively increases heart rate (McKitrick and Calaresu, 1991) and plasma levels of adrenaline and noradrenaline (Yashpal et al., 1985). A dense innervation of some IML neurons by SP-containing fibers has also been observed.

The predominant SP input to SPN's arises from bulbospinal projection neurons in the raphe oscurus, pallidus and n. interfascicularis hypoglossi. On the other hand, although ultrastructural evidence for direct SP inputs to retrogradely labelled SPNs has been demonsrated (Bacon and Smith, 1988; Vera et al., 1990), recent studies indicate that only a small subpopulation of SPNs receive SP inputs (Pilowsky et al., 1992), which is consistent with our observation that only a subpopulation of SPN's express the SP receptor. To determine whether functionally distinct populations of SPN express the SPR we are studying the expression of the SPR on SPN neurons labelled by transganglionic transport of pseudorabies virus from different peripheral targets.

The ventral horn:

Numerous studies have demonstrated supraspinally derived substance P in the ventral horn and electron microscopic studies have identified SP-immunoreactive inputs to spinal motoneurons (de Lanerolle et al., 1982; Vacca et al., 1983). Furthermore, iontophoresis of SP modifies the responses of motoneurons *in vivo* (Krivoy et al., 1980), and excites them after bath application *in vitro* (Otsuka and Yanagisawa, 1980). Despite thse observations, we found rather limited expression of the receptor in the ventral horn. Our results, however, are

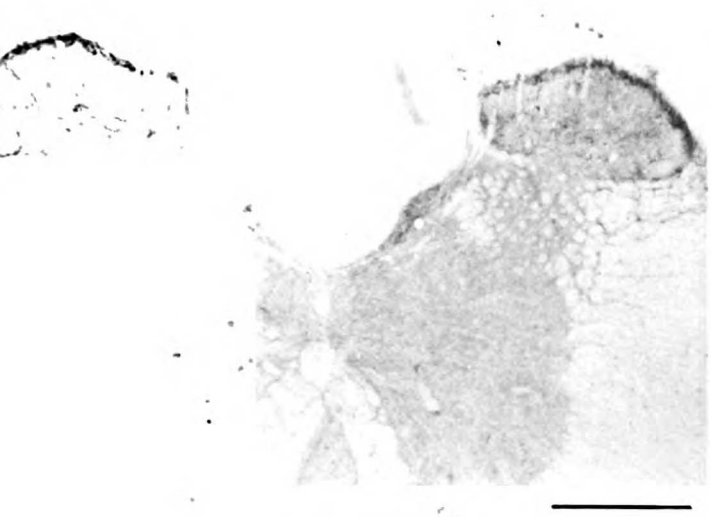
consistent with binding studies that reported only limited binding with NK-1 agonists in the ventral horn of the rat (Mantyh et al., 1989). When we localized SPR immunoreactivity in the ventral horn, it was generally diffuse, outlining motoneuron pools, but not defining the dendritic arbor of motoneurons with the fidelity comparable to that seen in the dorsal horn.

A striking exception to the sparse labelling in the ventral horn was the very dense labelling of neurons of Onuf's nucleus, which contains motoneurons that innervate the somatic musculature of the bladder. Since hemisection of the spinal cord, which eliminates SP from supraspinal sources, does not alter the level of SP around motoneurons in Onuf's nucleus (Rajaofetra et al., 1992; Tashiro et al., 1989), and since small diameter primary afferents fibers do not arborize in the ventral horn, it is likely that the SP input to this region derives from intraspinal sources. On the other hand, it is likely that there is a direct primary afferent derived SP input to SPR-immunoreactive parasympathetic preganglionic neurons of the sacral spinal cord. The inhibition of the micturition reflex by intrathecal injection of CP-96,345, a selective SP antagonist (Lecci et al., 1993), could thus result from blockade of both interneuronal and primary afferent-derived SP inputs to somatic and autonomic neurons that regulate bladder function.

Summary

In summary, we have demonstrated that the SPR receptor is expressed over almost the entire somatic and dendritic surface of subpopulations of neurons in the spinal cord and trigeminal nucleus caudalis. Importantly, morphologically distinct groups of SPR-immunoreactive neurons were readily recognized. Although the majority of the labelled neurons are located in regions that contain nociresponsive neurons (lamina I, V, VII and X), the presence of SPR immunoreactive antenna cells in lamina III suggests that nociceptive SP-containing primary afferent may regulate the firing of non-nociceptive as well as nociceptive neurons. The near absence of SPR immunoreactive interneurons in the SG, where SP terminals are most abundant, provides important evidence confirming the mismatch of peptides and peptide receptors. These results are consistent with the suggestion that SP acts in a "non-

synaptic" fashion on dorsal horn neurons. By combining anatomical markers of neuronal activity (e.g. *c-fos* immunocytochemistry) with SPR-staining, we are presently addressing the differential activation of SPR immunoreactive neurons by modality specific noxious stimuli.



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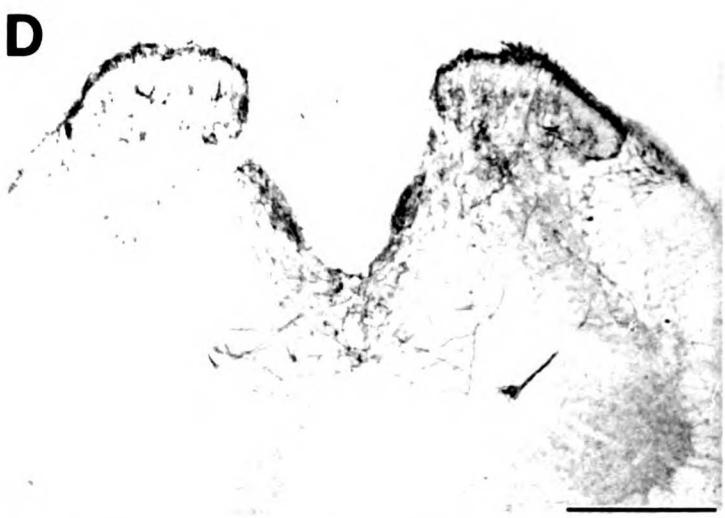

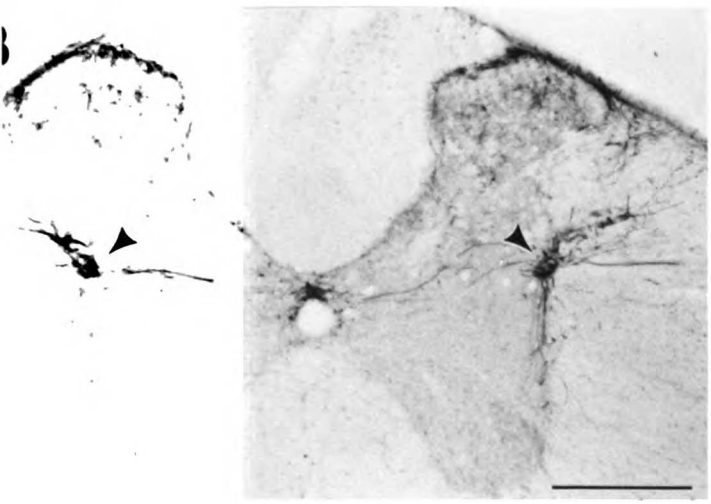

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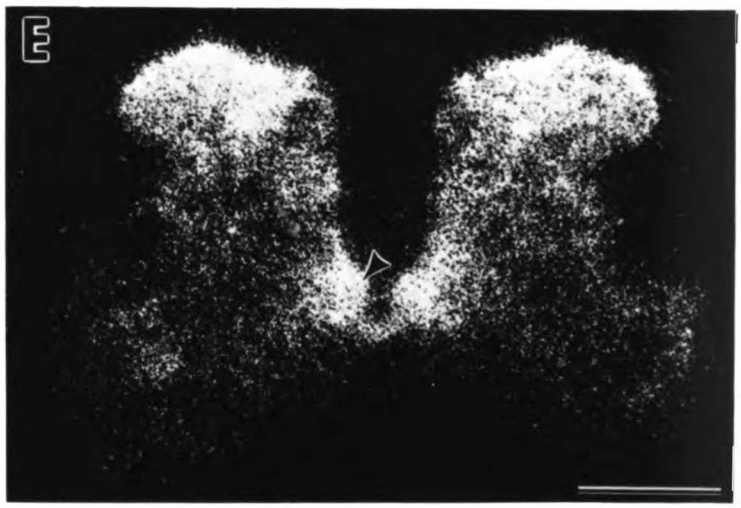

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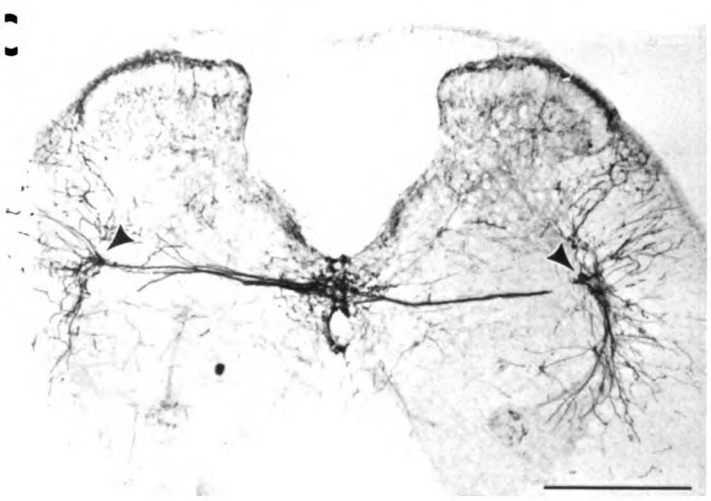

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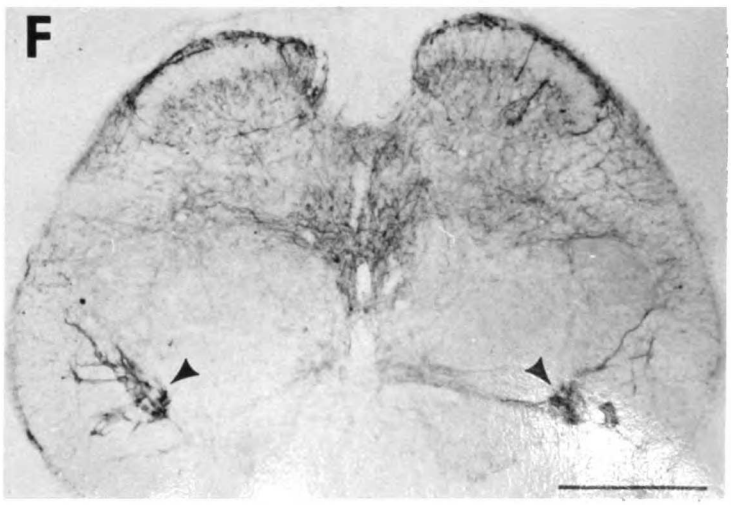


Figure 2. These photomicrographs illustrate the morphology and distribution of SPR-immunoreactive neurons in the superficial laminae of the lumbar dorsal horn. A: There is intense labelling in lamina I and a paucity of staining in the substantia gelatinosa (SG; coronal section). Arrowheads point to labelled dendrites that extend through the medial portion of the SG, to lamina I. B: SPR-immunoreactive Waldeyer neuron (arrowhead) in a transverse section through lamina I. C: This sagittal section illustrates many labelled marginal neurons in the most lateral part of lamina I, as it curves around the dorsal horn. D: SPR-immunoreactive lamina I neuron with ventrally-directed dendrites (arrowhead; coronal section). E: This sagittal section reveals that the majority of SPR-immunoreactive marginal neurons have long rostrocaudally oriented dendrites. Sagittal sections F and G illustrate examples of the rare SG SPR-immunoreactive neuron; these are large multipolar neurons with dendrites that arborize in lamina I and ventrally. Calibration bars equal 100 μm in A-G.

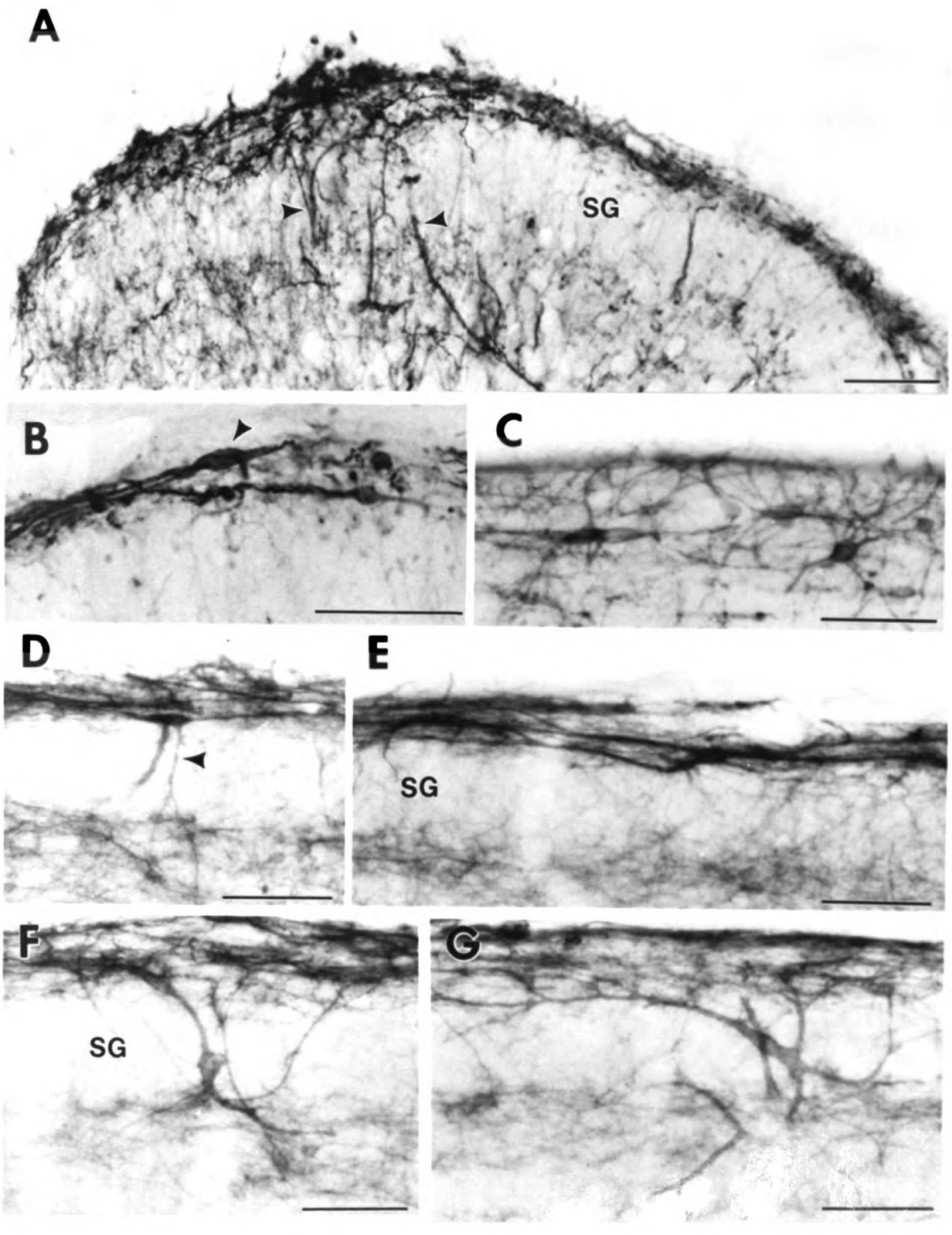

A
SG
B
C
D
E
SG
F
SG
G

Figure 3. These photomicrographs illustrate the morphology of SPR-immunoreactive neurons in lamina III and lamina IV of the lumbar dorsal horn. The large, multipolar "antenna" (Rethelyi and Szentagothai, 1973) neurons are best viewed in sagittal section (A,B). These have several dorsally directed dendrites that course through the SG and reach lamina I (arrowheads). Pyramidal cells can also be seen in sagittal section (C). Some of the dendrites are spine laden (C). The majority of the SPR-immunoreactive neurons in laminae III and IV are small and lightly labelled (arrowheads in D); their dendrites arborize within the nucleus proprius. Calibration bar equals 100 μm in A-D.

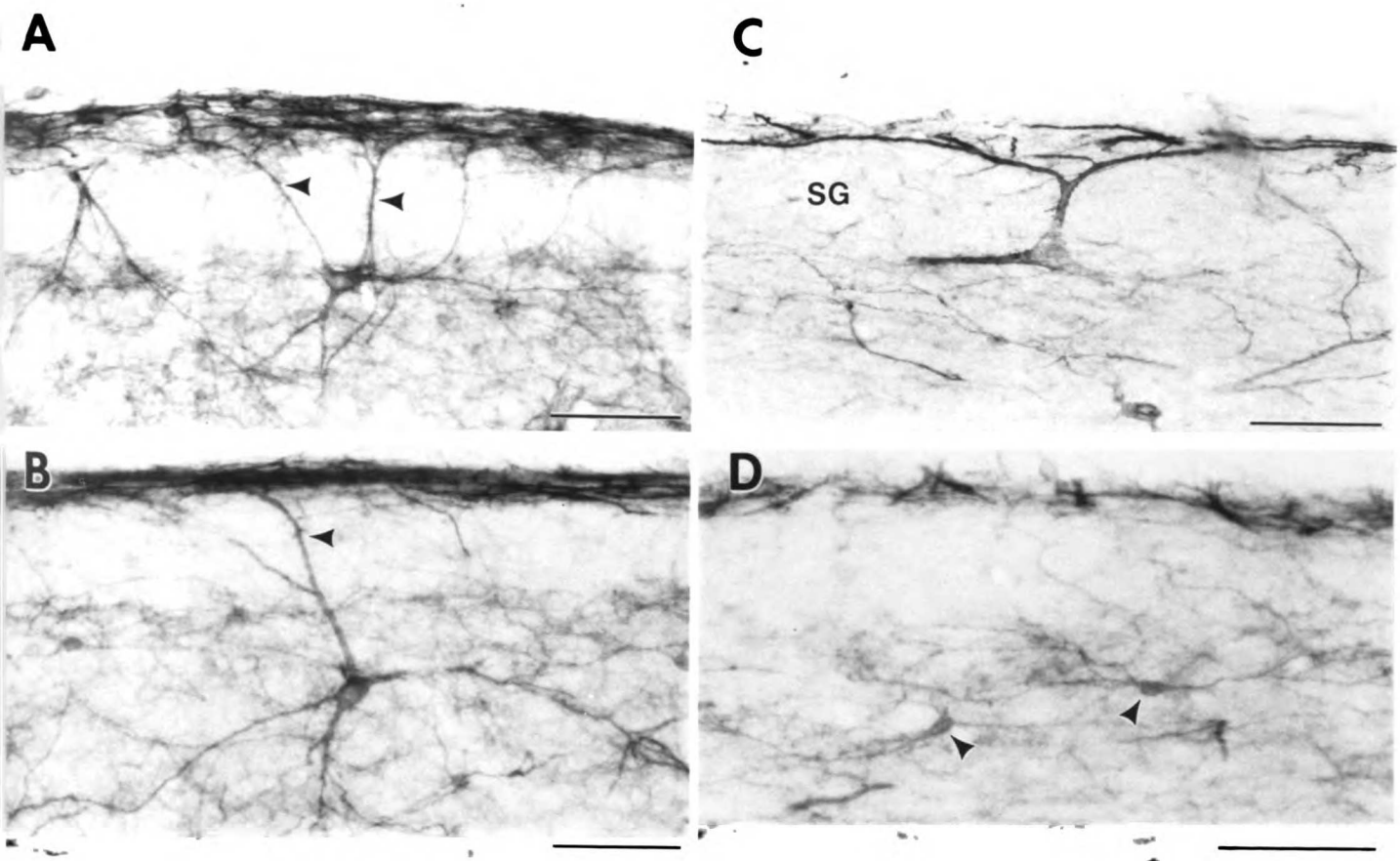

A
C
SG
B
D

Figure 4. These photomicrographs of transverse sections of the lumbar spinal cord illustrate the morphology of SPR-immunoreactive neurons in the neck of the dorsal horn (lamaine V and VI). Although some of the large multipolar neurons in this region have dendrites that penetrate the SG (A), the majority have dendrites that arborize extensively within the nucleus proprius, stopping at the ventral border of the SG (arrowhead in B). There are also many smaller fusiform neurons located along the medial border of the dorsal horn (C); fine processes emanating from these neurons often penetrate the medially adjacent dorsal column white matter (DC; arrowhead). The higher power images in D and E illustrate examples of the medially-located SPR-immunoreactive neurons in the neck of the dorsal horn (transverse section). Calibration bars equal 200 μm in A-C and 100 μm in D and E.

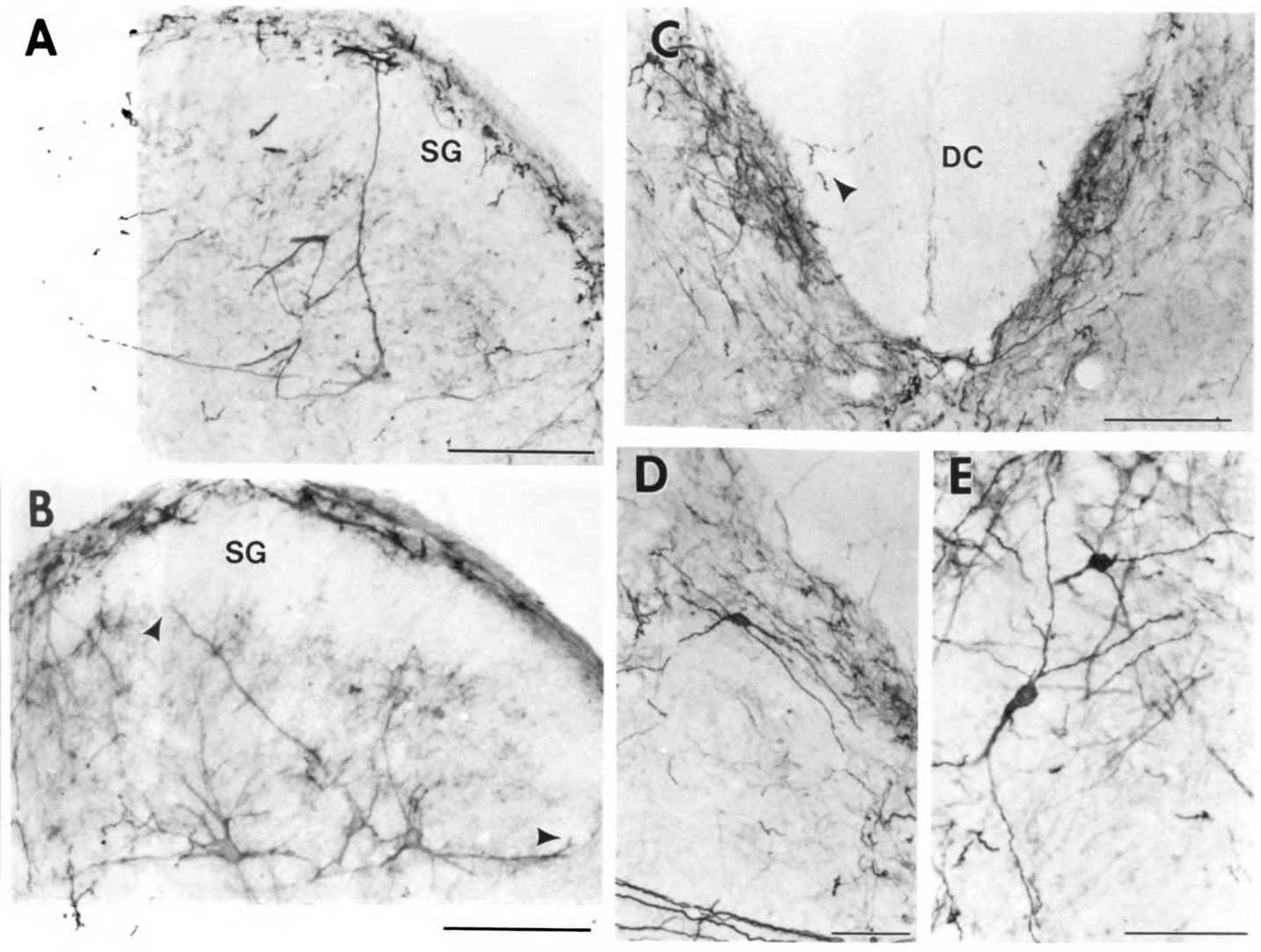

A
SG
B
SG
C
DC
D
E

Figure 5. These photomicrographs illustrate SPR-immunoreactive neurons in the IML (A) and in the ventral horn (B,C) of the lumbar cord. Many of the IML neurons have dendrites (arrowhead in A) that extend through the dorsolateral white matter to the lateral spinal nucleus (LSN), subjacent to the dorsal horn (dh). Some of the LSN neurons are also SPR-immunoreactive. The labelled dendritic bundles (arrowheads) that arise from SPR-immunoreactive neurons in the ventral horn can be seen in both transverse (B) and sagittal (C) sections. Calibration bars equal 150 μm in A and 200 μm in B and C.

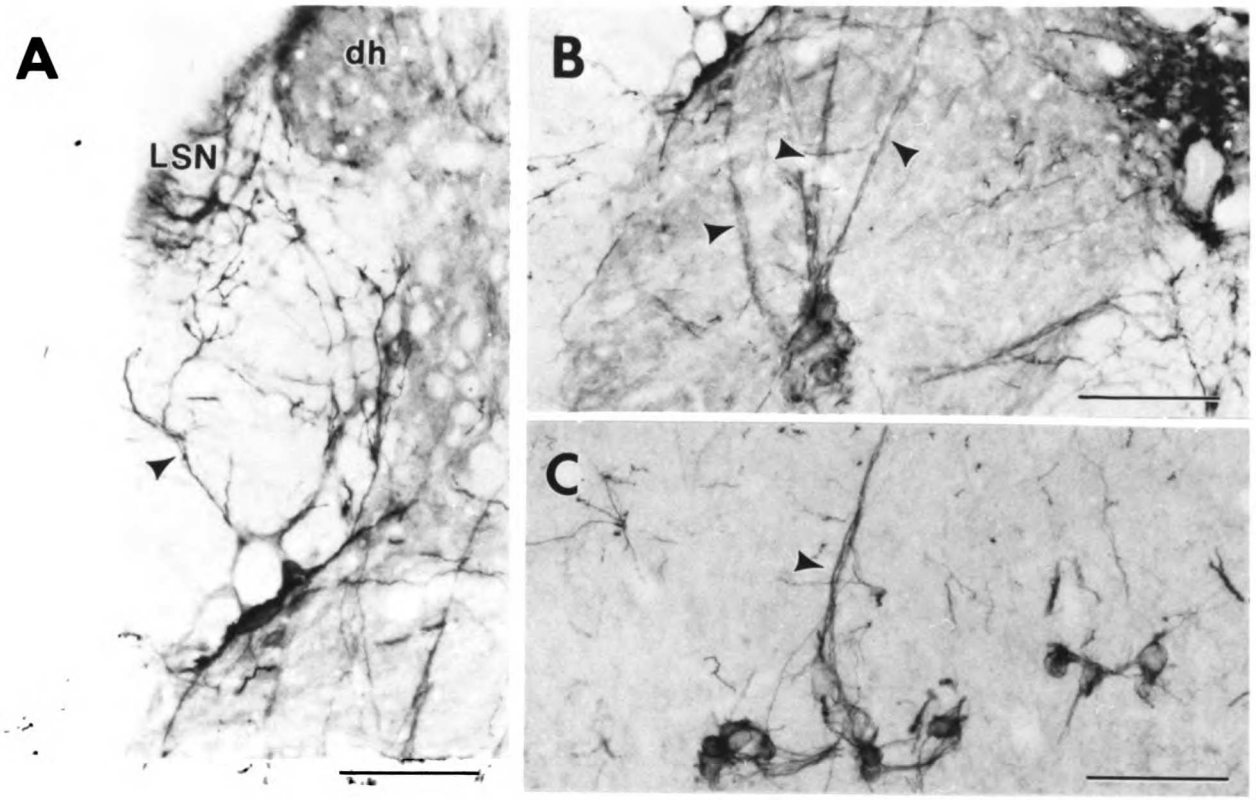
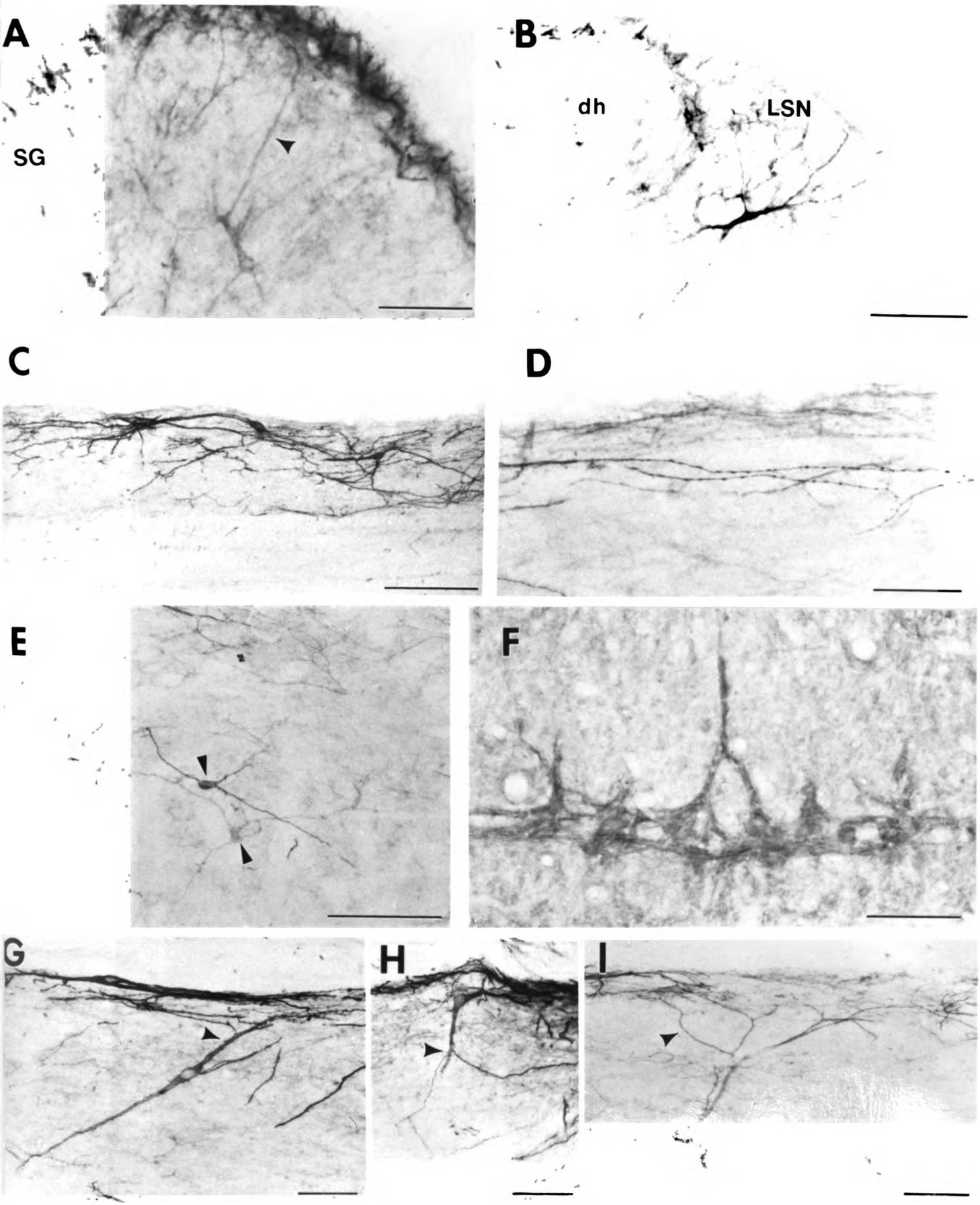

A
dh
LSN
B
C

Figure 6. These photomicrographs illustrate features of SPR-immunoreactive neurons in the cervical spinal cord. Dense staining in lamina I, the paucity of staining in the SG and the presence of a small population of lamina III multipolar neurons with dorsally directed dendrites (arrowheads) are seen in transverse section (A). Unusually large SPR-immunoreactive neurons in the LSN, subjacent to the dorsal horn (dh), are illustrated in B (transverse section). Sagittal sections (C,D and H) reveal additional features of the staining in the superficial dorsal horn. Typical marginal neurons, with rostrocaudally directed dendrites are illustrated in C. Occasionally thin beaded dendrites arise from more proximal dendritic branches of SPR-immunoreactive marginal neurons (D). Other marginal neurons have ventrally directed dendrites (arrowheads in H). The variety of morphologies of laminae III-IV neurons are illustrated in the sagittal sections G and I. As in the lumbar spinal cord, many of these neurons have prominent dorsally-directed dendrites (arrowheads). Lamina III also contains many small SPR-immunoreactive neurons whose dendrites remain in the deeper laminae (E). Finally, the dense labelling of subpopulations of motoneurons with veils of SPR immunoreactivity is illustrated in F. Calibration bars equal 100 μm in A, B, G-I: 200 μm in C, E and F and 50 μm in D.


A
SG
B
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LSN
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F
G
H
I

Figure 7. These photomicrographs illustrate SPR-immunoreactive sympathetic preganglionic neurons (SPN) in the thoracic spinal cord. Many IML neurons are very densely stained and have dendrites that extend well into the lateral white matter (arrowheads in A). The clustering of SPR-immunoreactive IML neurons is readily seen in sagittal section (B). The montage in C shows SPR-immunoreactive neurons of the IML, the intercalated (I) and central autonomic nuclei (CA). Neurons of the latter region have small cell bodies that are located dorsal to the central canal. Both IML and intercalated neurons have dendrites that extend to the central canal (arrowheads). Calibration bars equal 200 μm in A-C.

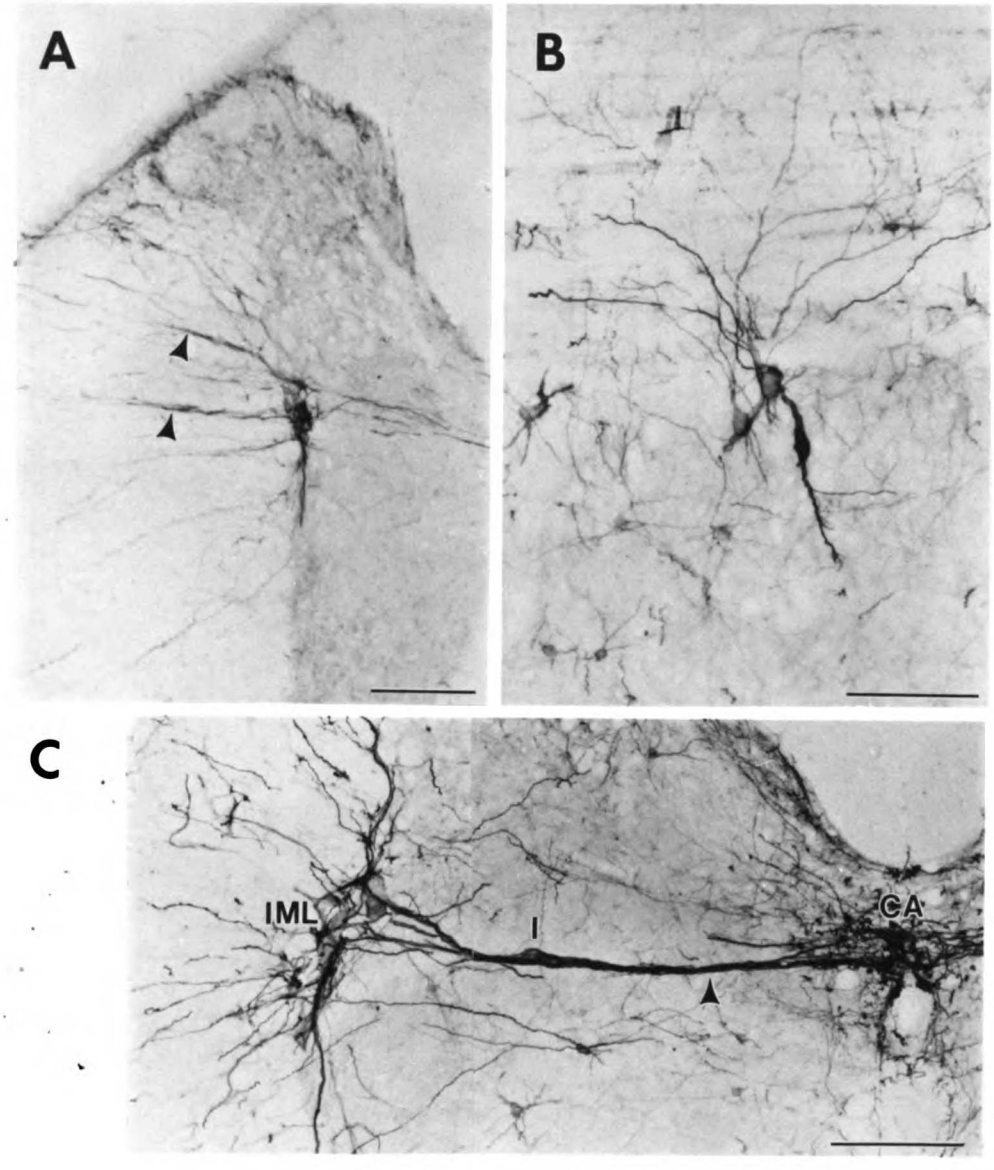

A
B
C
IML
I
CA

Figure 8. These photomicrographs illustrate the morphology and distribution of SPR-immunoreactive neurons in the sacral spinal cord. Lamina I is densely stained and there is a lack of staining in the SG (A, B). Large multipolar neurons in laminae III-V have extensive dendritic arbors; some of the dendrites cross the midline (arrowheads in A and B). There is also considerable labelling of presumed preganglionic parasympathetic neurons in the region of the sacral autonomic nucleus (C). Dendrites of these neurons also arborize extensively in the lateral funiculus and medially in the intermediate gray matter (arrowheads). Intense and diffuse staining of SPR-immunoreactive neurons in Onuf's nucleus (D). Bundles of labelled dendrites (arrowheads) course from the center of the nucleus. Calibration bars equal 300 μm in A and 200 μm in B-D.

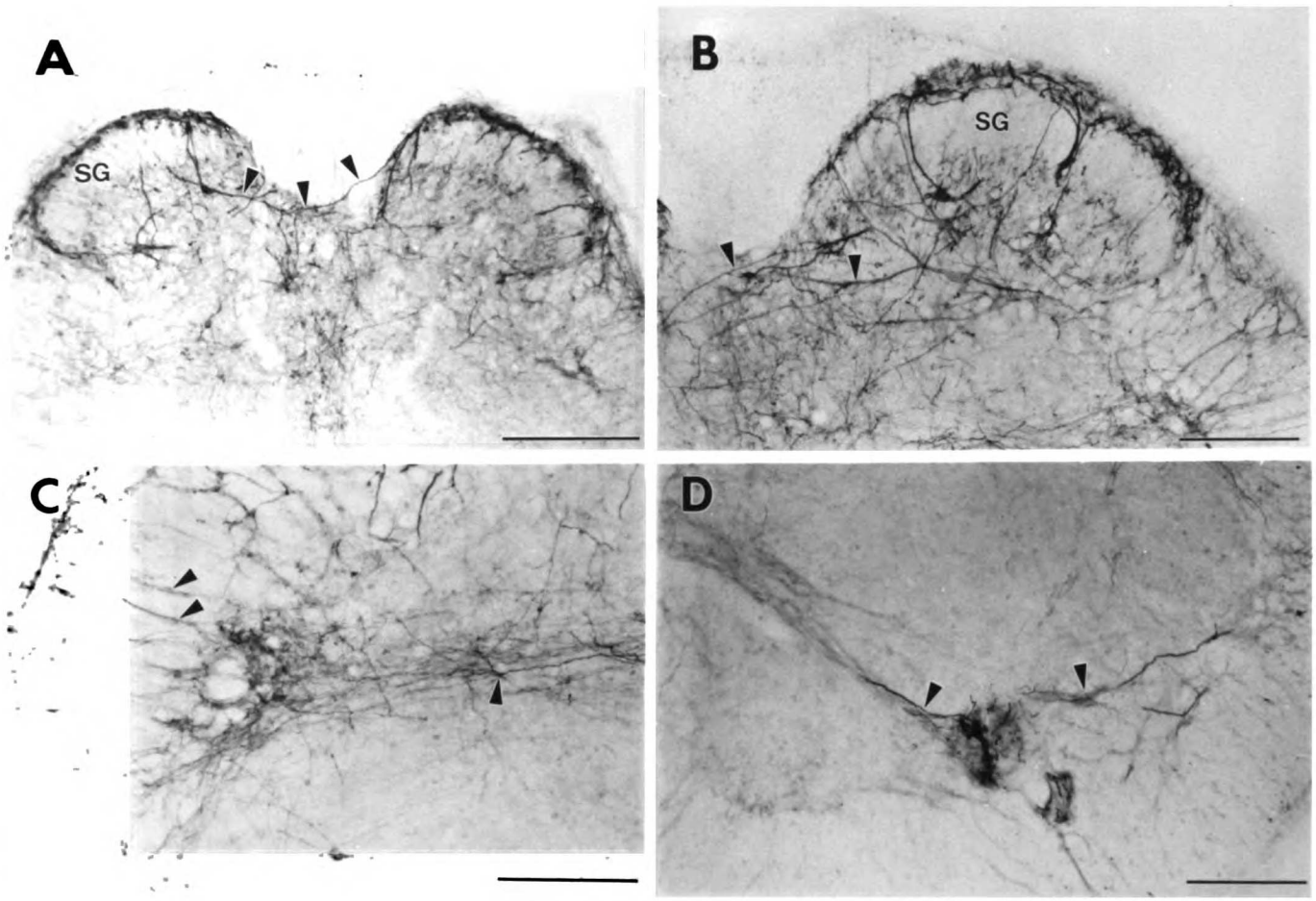
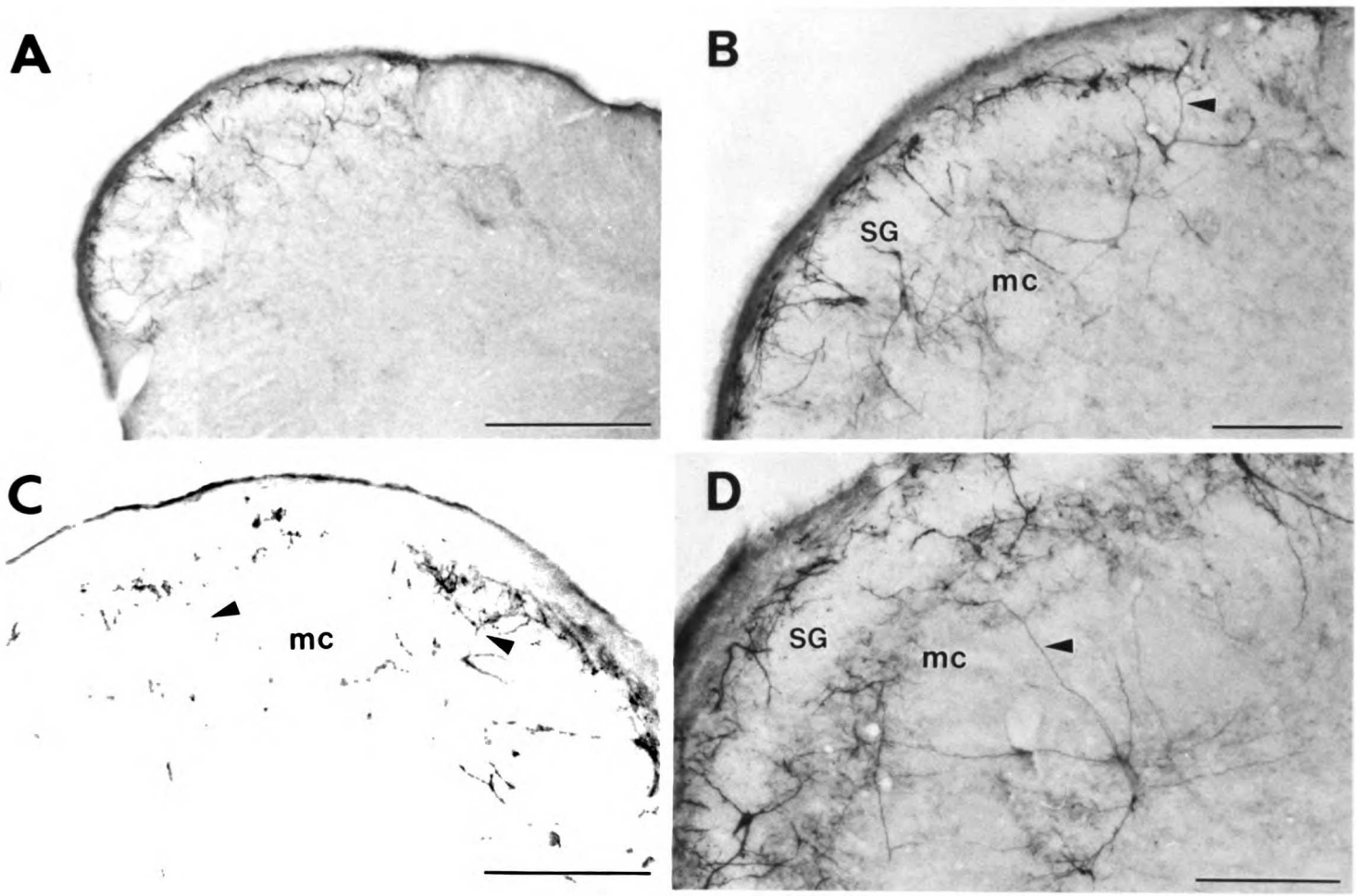

A
SG
B
SG
C
D

Figure 9. These photomicrographs illustrate the pattern of SPR immunoreactivity in the trigeminal nucleus caudalis. As in the spinal cord, there is dense staining in lamina I and only isolated dendritic labelling in the SG (A-D). Large multipolar neurons are located ventral to the SG, in the dorsal part of the nucleus magnocellularis (mc), which corresponds to laminae III and IV in the spinal cord. The dendrites of these neurons often coursed dorsally, through the SG (arrowheads in B,C). Occasionally we recorded very large SPR-immunoreactive neurons with cell bodies in the region of lamina V of the nucleus caudalis (D). The dendrites of the latter neurons often extended up to, but did not penetrate, the SG (arrowhead in D). Calibration bars equal 400 μm in A, 100 μm in B and 200 μm in C and D.


A
B
SG
mc
C
mc
D
SG
mc

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CHAPTER V

A subset of substance P receptor-immunoreactive neurons in the spinal cord project to supraspinal sites implicated in nociceptive transmission

INTRODUCTION

In the previous chapters we demonstrated that the densest substance P receptor labelling in the spinal cord was of cell bodies and dendrites in lamina I of the dorsal horn. As described above, many lamina I neurons receive a monosynaptic input from nociceptive primary afferent fibers, respond to noxious stimulation and are at the origin of major ascending pathways that have been implicated in the transmission of nociceptive information. In addition to the traditional thalamic targets, recent studies have emphasized the contribution of the spinomesencephalic, spinoparabrachial and spinosolitary pathways in the transmission of nociception messages (Bernard and Besson, 1992). There are also important projections to the subnuclei within the ventrolateral medulla that arise from neurons in lamina I. Morphological studies that examine the distribution of lamina I neurons that project to the parabrachial nuclei and to the nucleus of the solitary tract (NTS) (Esteves et al., 1993; Lima and Coimbra, 1989) revealed neurons that resemble the SPR-immunoreactive lamina I neurons. This suggests that at least some of the SPR-immunoreactive neurons are at the origin of ascending pathways, and thus might contribute to the rostral transmission of nociceptive information. On the other hand, since our cell counts revealed that SPR-immunoreactive neurons only account for about 5% of the total neuronal population in lamina I, without definitive studies, we cannot conclude that SPR-immunoreactive neurons are, in fact, a subpopulation of the lamina I projection neurons.

In the present study, we examined whether the SPR is expressed in spinal cord neurons that project to the brain. To this end, we used a double-labelling method to localize the SPR in spinal cord that were retrogradely labelled after tracer injections into the thalamus, parabrachial nuclei or NTS. We also evaluated the possibility that SPR-immunoreactive neurons in lamina I are at the origin of

propriospinal axons. Since the SPR-immunoreactive neurons in lamina I have fusiform cell bodies with long rostrocaudal directed dendrites, these were examined in the sagittal sections of lumbar cord. To accurately monitor the SPR-immunoreactive projection neurons found in the deeper spinal cord laminae, we analysed transverse sections of sacral cord, an area that contains a higher number of SPR-immunoreactive neurons in the nucleus proprius and in the neck of the dorsal horn compared to other segmental levels. For the thalamic and propriospinal projection neurons, which were largely found in the deeper dorsal horn laminae, we examined only transverse sections of the lumbar and sacral spinal cord.

METHODS

Protocol

Experiments were performed on 27 adult, male Sprague-Dawley rats (300 grams). Surgery and injections of the retrograde tracer were performed on animals deeply anesthetized with ketamine (60 mg/kg) and xylazine (6.0 mg/kg) with supplementation as necessary. The rats were placed in a stereotaxic frame and a hole drilled in the skull over the appropriate target. Pressure injections of the retrograde tracer, Fluorogold (FG) were made through glass micropipettes (tip diameter, ~ 40 μm) that were secured to a Hamilton syringe. The injections were targeted at one of three supraspinal sites: the parabrachial nucleus (PB), the nucleus of the solitary tract (NTS), the ventrobasal thalamus. In the ventrobasal thalamus we made four 0.04μl injections along two micropipette penetrations. For the other two sites, we made a single injection 0.06 μl, on one side. In addition, five animals received unilateral injections of the regrograde tracer into the cervical spinal cord in order to examine possible propriospinal neurons that expressed the SPR. To this end we performed a laminectomy over the cervical enlargement. After the dura was incised, we made injections of 0.05 μl, using a handheld 1.0 μl Hamilton syringe; the injections were made unilaterally, in order to fill the

dorsoventral extent a cervical segment. In these studies the experiments were designed to address the possible projection of neurons in laminae III/IV. The analysis was performed in the lumbar spinal cord, in transverse sections.

Although FG can be visualized with fluorescence microscopy, we opted to localize the tracer by immunoctochemistry, because subsequent double labelling of the SPR proved more reliable and produced a permanent reaction product that was more readily analyzed. Seven days after the FG injection the animals were reanesthetized with ketamine (100 mg/kg) and xylazine (7.0mg/kg) and intracardially perfused with 50 ml 0.5 M phosphate buffered saline (PBS), pH 7.4 at 37°C followed by 500 ml 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The spinal cords were removed and postfixed for 5 hours in the same fixative. The tissues were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer for two days at 4°C. Using a freezing microtome, we collected 35 µm transverse sections through the sacral enlargement; these were used for the analysis of SPR-immunoreactive projection neurons in deep laminae of the dorsal horn. The lumbar spinal cord was sectioned sagittally at 25 µm and used for the analysis of SPR-immunoreactive projection neurons in lamina I. The latter approach was used for lamina I because of characteristic rostrocaudal orientiation of the SPR-immunoreactive dendrites in lamina I. To visualize the injection sites, we sectioned the brain transversely at 50 µm.

For double labelling of FG and the substance P receptor, we used a two step avidin-biotin procedure (Hsu et al., 1981). The SPR was first visualized with a brown/black chromogen and then the FG was visualized with a red chromagen. Double labelled neurons had a red/pink cytoplasm (in cell bodies and proximal dendrites) that was surrounded by a brown/black SPR-immunoreaction product on the cell surface. In brief, spinal cord sections were incubated overnight at room temperature in a rabbit polyclonal primary antiserum directed against the C-terminal

portion of the substance P receptor (1:20,000; courtesy of Stephen Vigna, Duke University). After incubation in the primary antiserum, the sections were sequentially incubated in biotinylated goat anti-rabbit IgG avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction product was visualized with hydrogen peroxide and diaminobenzidine (DAB) as the chromogen, with nickel intensification (Ni-DAB). Following SPR immunocytochemistry, a similar process was repeated to visualize the FG. In this case, the SPR reacted tissue was incubated with a polyclonal antiserum directed against FG (1:30,000, Chemicon). After incubation in the primary FG antibody, the sections were incubated in a biotinylated goat anti-rabbit IgG followed by an avidin-biotin-alkaline phosphatase complex (Vector Laboratories, Burlingame, CA). The product of the alkaline phosphatase is red/pink in color, which is readily distinguished from the Ni-DAB reaction product. Although the latter is usually black, the sequential immunocytochemical protocols often resulted in a lightening of SPR immunoreaction product, to brown. After the second immunostaining protocol, the sections were mounted on gelatin coated Superfrost slides, which were later dehydrated and coverslipped.

Double labeled neurons were quantified by counting the number of SPR-FG immunoreactive double-labelled neurons as a percentage of the total number FG neurons or the total number of SPR-immunoreactive neurons. Results are given in percentages of double labeled neurons.

RESULTS

Distribution of ascending tract neurons in the spinal cord

Injections with the retrograde tracer fluorogold (FG) produced an overall pattern of retrogradely labeled spinal cord neurons that was consistent with previous reports. FG injections into the PB and NTS resulted in retrogradely labeled neurons in lamina I and the lateral spinal nucleus (LSN) of the lumbar spinal

cord; the majority of projection neurons labelled by the PB injections were located contralateral to the site of injection. A small number of spinal cord neurons in the neck of the dorsal horn were retrogradely labeled after FG injections in the ventrobasal thalamus; laminae III-IV neurons in the lumbar cord were predominantly labeled after FG injections into the cervical spinal cord. Since FG may be taken up by axons of passage as well as by axon terminals, we could not distinguish between propriospinal neurons and neurons at the origin of the spinocervical tract.

SPR-immunoreactive projection neurons

As described above, the lumbar and sacral segments of the spinal cord contained a greater density of neurons that expressed the substance P receptor (SPR) compared to either the cervical or thoracic levels. Because of this, we confined our examination of the SPR-immunoreactive projection neurons to the lumbar and sacral levels.

Projections to parabrachial nucleus

Retrogradely labeled spinal cord neurons that resulted from injections centered on the PB were largely found in lamina I (Figure 1A). It is our impression that the largest number of neurons were located in the most outer edge of lamina I, as seen in transverse sections of the sacral cord (Figure 1A). Analysis of labeled neurons in lamina I from sagittal sections of the lumbar cord revealed that of the total marginal cell population that has been retrogradely labeled, 12% of these neurons express the SPR (Figure 1B, 1C). In contrast, we observed that of the total population of SPR-immunoreactive neurons in the marginal layer, 31% were projection neurons to the PB. Thus, while a large proportion of SPR-immunoreactive neurons project to the PB, overall this group of SPR-immunoreactive projection neurons comprises a relatively small proportion of the total ascending projection to the PB.

Projections to nucleus of the solitary tract

Retrogradely labeled spinal cord neurons that resulted from injections centered on the NTS were largely found in lamina I. In this case, we often observed labeled neurons in deeper dorsal horn laminae; however, these labeled neurons were due to spread of FG to structures adjacent to the NTS, including the dorsal columns. This was determined from control injections of FG that were targetted in the dorsal column nuclei. The latter resulted in labelling of neurons exclusively in deeper laminae.

As with PB projection neurons in lamina I, we also observed that retrogradely labeled neurons from the NTS were largely confined to the outer edge of lamina I, as seen in transverse section (Figure 2A). Analysis of labeled neurons in lamina I from sagittal sections of the lumbar cord (Figure 2B) established that of the total marginal cell population that has been retrogradely labeled, 26% of these neurons express the SPR. In contrast, we observed that of the total population of SPR-immunoreactive neurons in the marginal layer, 41% were projection neurons to the NTS. Thus, we find again that a large proportion of SPR-immunoreactive neurons project to the NTS; and in addition, this SPR-immunoreactive projection comprises a moderate proportion of the total ascending pathway to the NTS.

Projections to ventrobasal thalamus

A small population of retrogradely labeled spinal cord neurons that resulted from injections centered on the ventrobasal thalamus were concentrated in lamina IV-V of the lumbar spinal cord. In this case, we did not find a subpopulation of SPR-immunoreactive neurons that were retrogradely labeled.

Propriospinal projections

Unilateral tracer injections in the cervical spinal cord resulted in the retrograde labeling of neurons in laminae III-VI of the lumbar cord (Figure 3A,B). We further found that a large proportion the retrogradely labeled neurons expressed

the SP receptor. In fact, analysis of only the SPR-immunoreactive neurons in laminae III and IV showed that 42% of SPR-immunoreactive neurons are propriospinal projection neurons, or are neurons at the origin of the spinocervical tract. Among the double labelled neurons were the very large SPR-immunoreactive antenna cells located in lamina III. Because the injection sites always completely filled one half of the cervical cord, it was not possible to determine whether the axons of the retrogradely labeled neurons terminated in either the dorsal or ventral horns.

DISCUSSION

The present study has established that a subpopulation of neurons that express the SP receptor in lamina I are projection neurons that terminate in brainstem regions involved in nociceptive processing. In addition, we also observed that the smaller population of SPR-immunoreactive neurons in laminae III and IV of the lumbar cord are sites of propriospinal projections.

SPR-immunoreactive neurons project to the PB

We distinguished a subpopulation of SPR-immunoreactive neurons in lamina I that comprised a small proportion of the projection to the parabrachial; these double-labeled neurons had fusiform somata and long rostrocaudally directed dendrites. A class of spinoparabrachial neurons, which respond to nociceptive inputs, are morphologically similar to the spinoparabrachial neurons found in this study (Lima and Coimbra, 1989). Further studies have shown that the spinoparabrachial neurons are an important link in the transmission of nociceptive information via the PB to the medial nucleus of the amygdala (Bernard and Besson, 1992; Bernard et al., 1994; Hylden et al., 1986; Light et al., 1993) From our findings it seems that a subpopulation of spinoparabrachial neurons are specifically driven by afferent-derived SP. Since most evidence on SP release agree that persistent noxious inputs that involve tissue injury evoke afferent derived SP release in the dorsal horn, especially in superficial laminae, suggests that the SPR-immunoreactive projection neurons may relay some aspect of persistent noxious stimulation to the PB.

SPR-immunoreactive neurons project to the NTS

A subpopulation of SPR-immunoreactive neurons in lamina I also project to the NTS; however, the SPR-immunoreactive neurons, in this case, provided a major component, approximately one-fourth of the total neurons, of the spinosolitary pathway. Morphological characterization of the spinosolitary tract

projection neurons is similar to the morphology of SPR-immunoreactive projection neurons in lamina I; these neurons have fusiform somata and long rostrocaudally directed dendrites (Esteves, et al., 1993). Evidence supports that marginal neurons respond to and transmit nociceptive inputs (Christensen and Perl, 1970). In addition, electrophysiological recordings reveal that marginal neurons also receive visceral and somatic inputs, which are transmitted via the spinosolitary tract to the NTS (Person, 1989; Takahashi and Yokota, 1983). Previous reports (Menetrey and Basbaum, 1987) have already suggested that the NTS serves as a site of integration for nociceptive and autonomic inputs that are transmitted via the lamina I projection neurons. Our findings of SPR-immunoreactive projection neurons in lamina I support this hypothesis and further suggests that an important component of this projection is driven by substance P.

SPR -immunoreactive propriospinal neurons

Although neurons in lamina III-IV are largely thought to process non-nociceptive information and do not directly receive primary afferent derived SP, we have documented a population of III-IV neurons that express the SPR. The morphology of the laminae III-IV SPR-immunoreactive neurons resembles that of the antenna neurons described from Golgi material (Maxwell et al., 1983); these neurons are large multipolar cells with long dendritic arbors extending dorsally to the superficial laminae. In the present study, we have confirmed that a large proportion of these SPR-immunoreactive neurons of lamina III-IV are propriospinal neurons projecting to cervical segments within the spinal cord. Although the majority of lamina III-IV neurons respond to non-noxious stimulation, some nociceptive neurons with similar morphology have been reported in these laminae (Fleetwood-Walker et al., 1990). In most of these reports, it was established through antidromic activation that these III-IV neurons were spinocervical tract neurons. Presumably, the nociceptive input to III-IV neurons derives from small

diameter primary afferent termination on the distal dendrites on these neurons. However, substantial evidence also supports the SP can diffuse from its site of release to act on more ventral structures. Thus, it is possible that we have neurochemically identified a subset of lamina III-IV neurons involved in the intergration of somatic and nociceptive inputs.

In summary, this study has established that spinal cord neurons that express the SP receptor are often cells of origin for ascending pathways. Specifically, these findings show that SPR-immunoreactive neurons in lamina I contribute to the spinoparabrachial and spinosolitary projections, two sites known to process nociceptive information. In addition, we identified SPR-immunoreactive neurons in laminae III-IV that contribute to propriospinal projections and are morphologically similar to a subset of nociresponsive neurons found in the nucleus proprius. These findings suggest that while SPR-immunoreactive neurons make up only a small portion of the total number of spinal cord neurons, they are functionally important in the transmission of SP mediated nociceptive signals in ascending pathways.

Figure 1. These photomicrographs illustrate the distribution of SPR-immunoreactive neurons and retrogradely labelled neurons produced by FG injection into the parabrachial nucleus. A: Transverse section through the sacral cord shows retrogradely labeled neurons (pink; arrowheads) in lamina I; the labelled neurons are concentrated along the outer edge of the marginal layer. Note the large SPR-immunoreactive neuron in lamina III that is not retrogradely labelled from the PB. B: Sagittal section of lamina I in the lumbar spinal cord shows two SPR-immunorective neurons (arrowheads) that project to the parabrachial nucleus. The photomicrograph also contains several retrogradely labelled neurons that are not SPR-immunoreactive. C: This sagittal section of lamina I of the lumbar cord contains four marginal neurons that are immunoreactive for the SPR and retrogradely with fluorogold from the PB. (sg-substantia gelatinosa; LSN-lateral spinal nucleus). Calibration bars equal 150 μm in A and 50 μm in B and C.

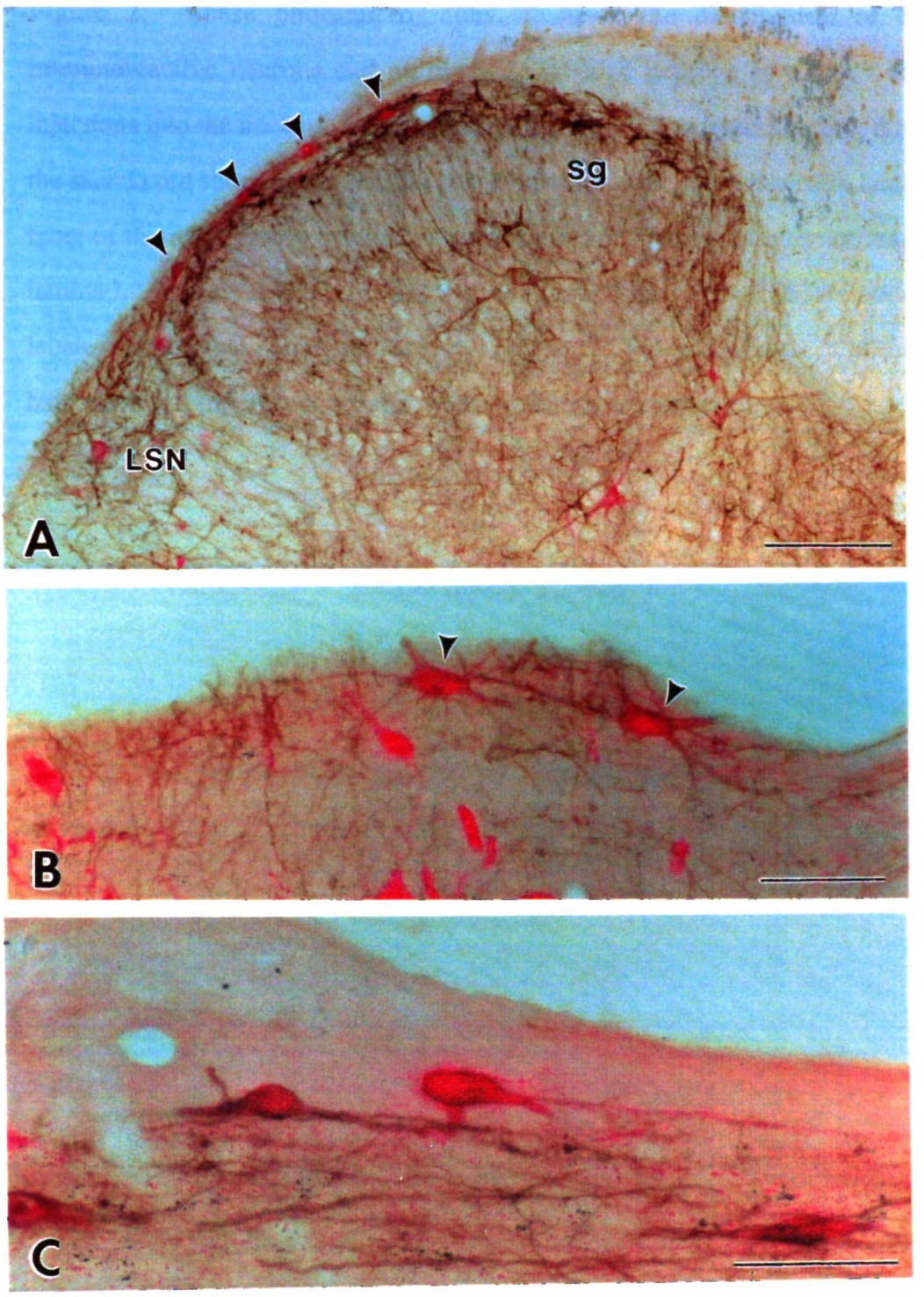
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Figure 2. These photomicrographs illustrate the distribution of SPR-immunoreactive neurons and retrogradely labelled neurons as a result of FG injections into the nucleus tractus solitarius (NTS). A: Transverse section through the sacral cord shows retrogradely labeled neurons (pink; arrowheads) in lamina I; most of the retrogradely labelled neurons are found along the outermost edge of lamina I. B: Sagittal section through lamina I of the lumbar cord shows a double-labeled neuron that is immunoreactive for SPR and FG (arrowhead). Calibration bars equal 100 μm in A and 50 μm in B. (sg, substantia gelatinosa).

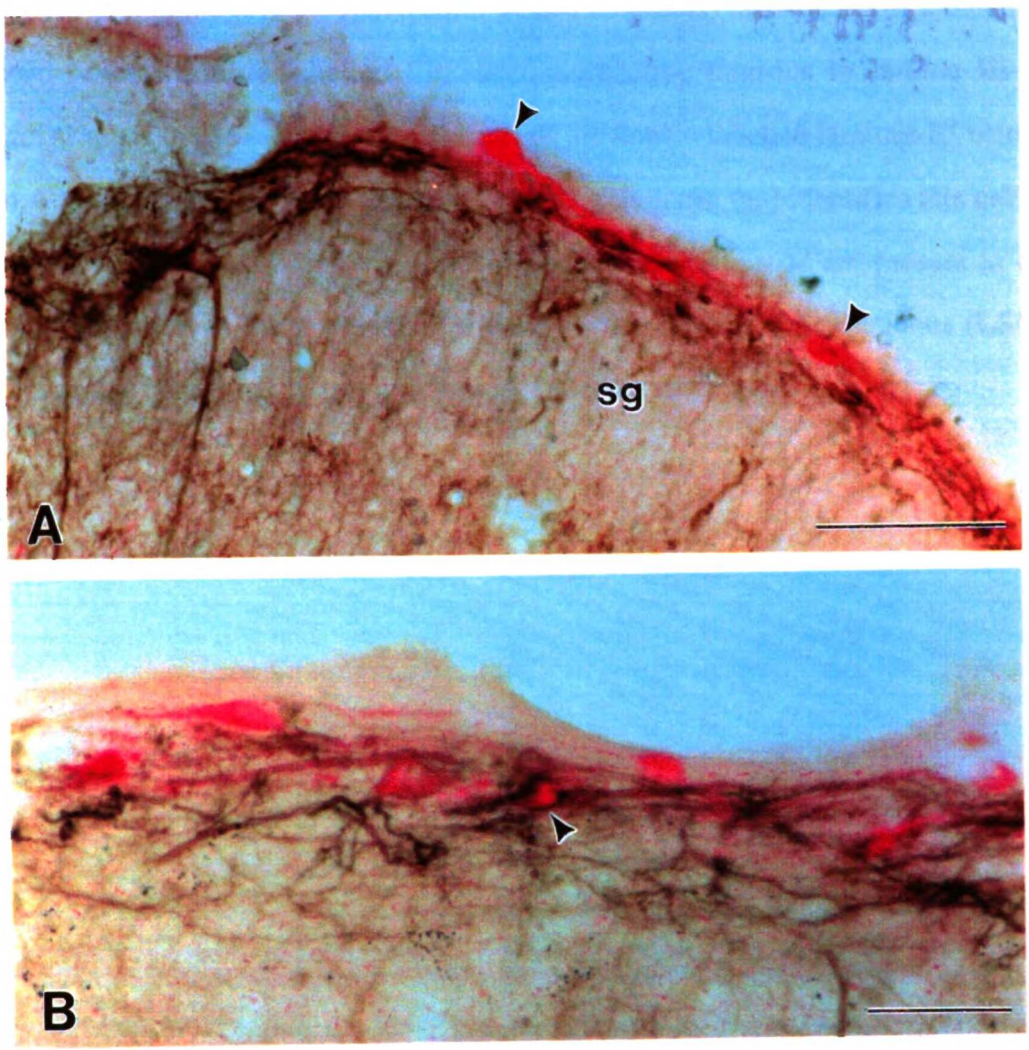
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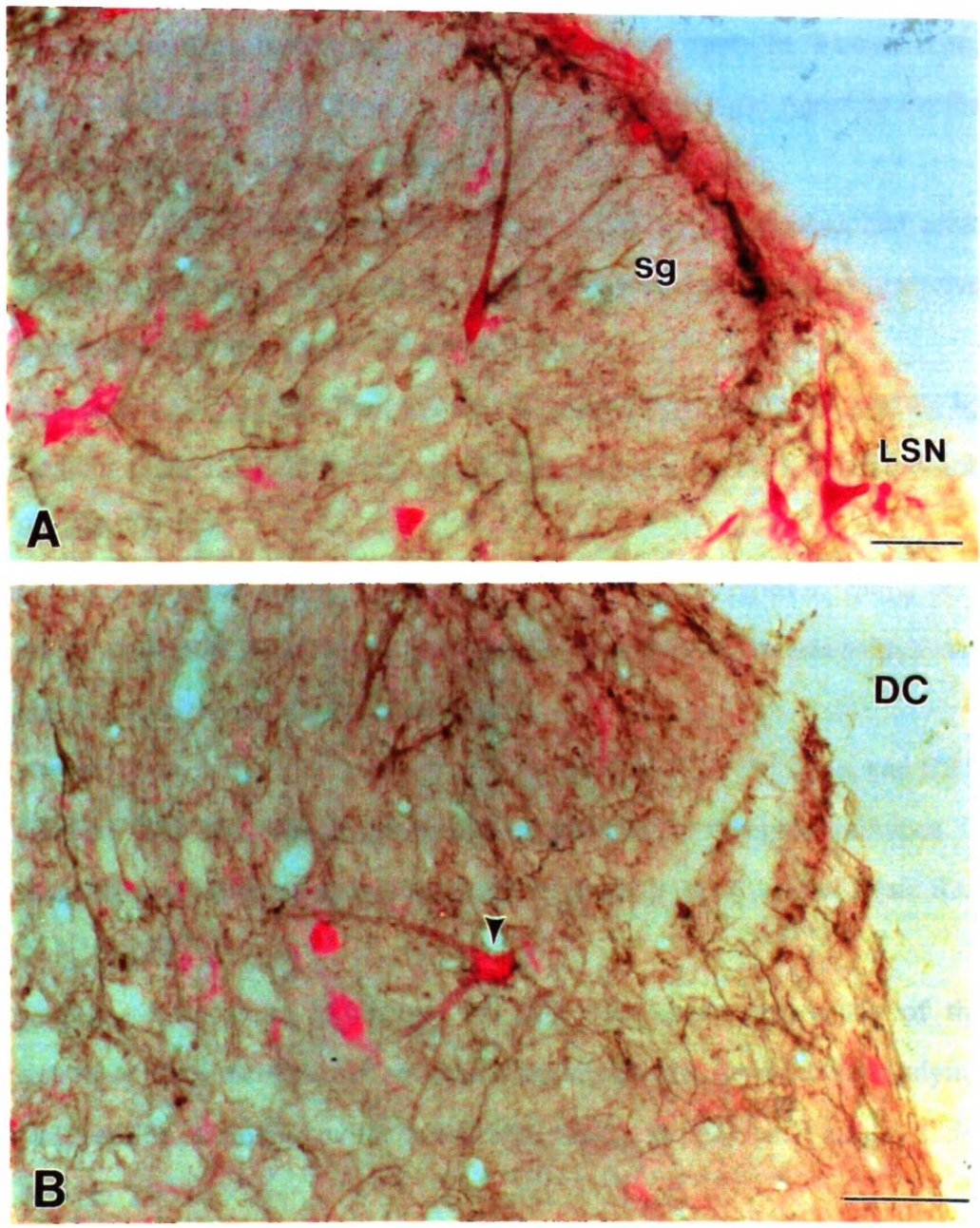
Figure 3. These photomicrographs of illustrate the distribution of SPR-immunoreactive neurons and retrogradely labelled neurons as a result of FG injections into the cervical spinal cord. A and B: Transverse sections of the lumbar cord reveals double labelled SPR-immunoreactive neurons in lamina III-IV (arrowheads). The fact that the dendrites of the double labelled laminae III neuron in A arborize dorsally, through the substantia gelatinosa (sg) identifies this cell as an antenna neuron. Many single labelled projection neurons are present in the marginal layer, in the nucleus proprius and in the lateral spinal nucleus (LSN). Calibration bars equal 50 μm in A and B. (DC; dorsal columns).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CHAPTER VI

Monitoring noxious stimulus evoked "activity" of substance P receptor-immunoreactive neurons: technical considerations

Jessica Brown and Allan Basbaum

INTRODUCTION

As several studies have established the utility of following the expression of the c-fos proto-oncogene to monitor the activity of large populations of neurons (Menetrey et al., 1989; Morgan et al., 1987; Morgan and Curran, 1991), we developed a paradigm to examine "activated" SPR-immunoreactive neurons that co-express the Fos protein. This examination required 1) the development of an appropriate double-labeling immunohistochemical procedure that would provide equal visualization of both the SPR and Fos proteins and 2) the development of a nociceptive behavioral paradigm that provides a significant percentage of SPR-immunoreactive neurons that co-express the Fos protein. The long term goal being that through pharmacological antagonism of Fos in the SPR-immunoreactive neurons, we might decipher the inputs that activate SPR-immunoreactive neurons. This report accounts the development of the paradigm used to examine the activity of the SPR-immunoreactive neurons in the spinal cord.

METHODS AND MATERIALS

Several immunocytochemical procedures were attempted to find the most suitable means of visualizing both Fos and SPR proteins. We first tried several permutations of the avidin-biotin procedure, described by Hsu et al (Hsu et al., 1981), which consists of an incubation in the primary antiserum, followed by an incubation in biotinylated goat anti-rabbit IgG, and finally incubated in an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction product can be visualized using hydrogen peroxide and diaminobenzidine (DAB) as the chromagen with or without nickel intensification. Initially, we used a double-labelling protocol previously used in our laboratory to visualize the first protein with diaminobenzedine (DAB), which produces a brown reaction product, and visualized the second protein with a nickel-enhanced diaminobenzedine (Ni-DAB)

that produces a black reaction product. However, several problems arose when this double labeling procedure was applied to the SPR/Fos visualization.

(1) Since both the Fos and SPR antibodies were made in the same species, double-labeling required that the tissue be fully processed through two rounds of immunocytochemistry. This increased wear and tear on the tissue. The increased damage to the tissue was especially prominent for membraneous proteins, like the SPR, and meant that for adequate visualization of the SPR-immunoreactive neurons, immunocytochemistry of the SPR must be performed *first.*

(2) Because of the density of SPR immunoreactivity along the neuronal membrane, we felt that it would be easier to identify double-labeled neurons using the DAB brown reaction product to visualize the SPR immunoreactivity and using the Ni-DAB black reaction product to visualize the Fos immunoreactivity. However, when the DAB brown product for the SPR immunoreactivity was run through the Ni-DAB reaction for visualization of the Fos, the DAB brown product was transformed into a deeper, darker brown, probably due to nickel deposits. This made identification of the double-labeled neurons difficult. Attempts to visualize the SPR immunoreactivity with Ni-DAB and the Fos immunoreactivity with DAB meant that the SPR Ni-DAB reaction had to be limited to produce a gray, instead of black, reaction product. However, the incomplete reactions of the SPR introduced a large amount of variabiltiy in the detection of SPR-immunoreactive neurons and was also an unsatisfactory method.

Failure to produce satisfactory double-labeling of the SPR and Fos proteins prompted our search for a different means of detection. To enhance contrast between the SPR and Fos immunoreactivities, we tried using a red chromogen (Vector Laboratories, Burlingame, CA) to visualize the fos protein within the Ni-DAB labeled SPR-immunoreactive neurons. This approach differs from DAB reactions. The red reaction product requires incubations in biotinylated goat anti-

rabbit IgG followed by an avidin-biotin-*alkalinephosphatase* complex (Vector Laboratories, Burlingame, CA). Using this protocol, fos-immunoreactive neurons contain pink or red nuclei, which is readily distinguishable from the brown/black SPR immunoreaction product. Fading of the black Ni-DAB reaction product to brown was often noted, but this did not interfere with differentiating SPR from Fos. The protocol used for double-labelling is given in full below.

Spinal cord sections were incubated overnight at room temperature in a rabbit polyclonal primary antiserum directed against the C-terminal portion of the substance P receptor (1:20,000; courtesy of Stephen Vigna, Duke University). After incubation in the primary antiserum, sections were sequentially incubated in biotinylated goat anti-rabbit IgG and an avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction product was visualized with hydrogen peroxide and diaminobenzidine (DAB) as the chromogen, with nickel intensification. Following SPR immunocytochemistry, a similar process was repeated to visualize Fos. In this case, the SPR reacted tissue is incubated with a polyclonal antiserum directed against an *in vitro* translated protein product of the *c-fos* gene (1:21,000: courtesy of Dennis Slamon, Department of Hematology and Oncology, UCLA). After incubation in the primary antibody, sections were sequentially incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-alkaline phosphatase complex (Vector Laboratories, Burlingame, CA). The fos protein thus visualized is a bright red reaction product. After the second immunoreaction, the sections were mounted on gelatin coated Superfrost slides, which were later dehydrated and coverslipped.

Behavioral models

Once the double-labelling procedure had been devised, we began testing for a suitable behavioral paradigm that would allow us to monitor the Fos "activity" of SPR-immunoreactive neurons. Male Sprague-Dawley rats (350-400 g; Bantin and

Kingman) were used in all studies. Because of the possible selective SP inputs that have been implicated in the different modalities of noxious stimulation, we tried three different modalities of noxious stimulation: mechanical, heat, and chemical stimulation. To help eliminate the additional somatic input that would result from the different behaviors, i.e. secondary to movement, the rats were maintained under a mixture of 2% halothane and 1% nitrous oxide anesthesia for the duration of behavioral testing. Rats tested with the noxious mechanical stimulus had the dorsal and ventral surfaces of the right hind paw clamped between an alligator clamp for a period of 30 seconds, every 2 minutes, for a duration of 20 minutes. The 50°C heat stimulus was applied by dipping the right hind paw in a beaker of 50°C water for a period of 30 seconds, every 2 minutes, for a duration of 20 minutes. The chemical irritant stimulus consisted of a subplantar injection of 5% formalin in a volume of 100 µl into the plantar surface of the right hind paw. The total time elapsed between initiation of noxious stimulus and killing of the animal was one hour and 20 minutes. After the test period, the rats were deeply anesthetized with ketamine (60 mg/kg) and xylazine (6.0 mg/kg) and intracardially perfused with 50 ml .05 M phosphate buffered saline (PBS) pH7.4 at 37°C followed by 4% formaldehyde in 0.1 M phosphate buffer pH 7.4. The spinal cords were removed and postfixed for 5 hours in the same fixative. The tissues were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer for two days at 4°C. Using a freezing microtome, we sectioned the spinal cords transversely at 40 µm.

Distribution of fos-like immunoreactive neurons and evidence for double-labeled neurons

Noxious stimulation, regardless of the modality, elicited a similar distribution of Fos like immunoreactivity in the dorsal horn of the lumbar spinal cord (Figure 1). Within two areas of Fos "activity," including lamina I and the medial portions of laminae IV and V, we found evidence of SPR-immunoreactive

neurons that co-expressed the fos immunoreactivity, regardless of the modality of noxious stimulus.

Lamina I

The highest density of Fos immunoreactive neurons appeared in laminae I and outer II, with a majority of these neurons concentrated in the medial portion of the laminae, which corresponds topographically to the location of termination of primary afferents from the hindpaw. A single 40 μm section showed an average of 40 fos-like immunoreactive neurons per section, regardless of the modality. Examination of the labelling in the superficial dorsal horn revealed that a small percentage of the fos like immunoreactivity was expressed within a subpopulation of SPR-immunoreactive neurons in lamina I (approximately 2 double-labeled neurons per section), again regardless of the modality. Although these numbers are small, it should be emphasized that the SPR-immunoreactive marginal neurons account for only 5% of the total marginal cell population, and in fact the 2 double-labeled neurons that we detect in transverse section would amount to about 5% of the fos-like immunoreactive neurons.

The fact that each of the three noxious stimulus modalities resulted in double labelling of a small population of marginal neurons provided no evidence for a selective modality activation of SPR-immunoreactive neurons in the dorsal horn. Given that our goal was to establish a behavioral paradigm that would allow us to monitor the "activity" of SPR-immunoreactive neurons, especially those in lamina I, it appeared that any of the three models tested would be adequate. Since there is more evidence (Kuraishi et al., 1989) for a selective contribution of SP to noxious mechanical stimulation, to further examine the "activation" of SPR-immunoreactive marginal neurons we elected to use the mechanical model.

Medial aspect of laminae IV and V

A small population of fos-like immunoreactive neurons was distributed

along the medial border of laminae IV-V; we recorded approximately 20 labeled neurons per section (Figure 1). Within this area, we observed that a small population of SPR-immunoreactive neurons, which were previously characterized as fusiform cells with dendritic branches directed dorsally and ventrally, were frequently double-labeled. Cell counts of this small population indicate that 67% of these SPR-immunoreactive neurons co-expressed Fos immunoreactivity following noxious stimulation, regardless of the modality (Figure 3A,B). Thus, again it appears that the modality of the noxious stimulation was not an important factor in "activating" the SPR-immunoreactive neurons along the medial border of laminae IV-V.

Other areas that contain fos-like immunoreactive neurons

Just ventrolateral to the superficial laminae in the area of the lateral spinal nucleus (LSN), we observed a small group of fos-like immunoreactive neurons. The LSN usually contained approximately 5 labeled neurons per section. There was evidence of double-labeled neurons, but it was not quantified. A second group of fos-like immunoreactive neurons was clustered around the central canal (CC) and on average consisted of 15 neurons per section. The third group consisted of a diffuse set of fos-like immunoreactive neurons located in the reticulated part of IV and V (average of 7 cells per section). There was no evidence of double labeling in these areas.

Further examination of the SPR-immunoreactive lamina I neurons

As discussed above, we elected to use a noxious mechanical stimulus to further examine the induction of fos in SPR-immunoreactive neurons of lamina I. Importantly, since the dendrites of the majority of SPR-immunoreactive marginal neurons arborize rostrocaudally, we studied this tissue in 25 μm sagittal sections. We found that approximately 30 % of the SPR-immunoreactive neurons in the medial half of lamina I are double-labeled after noxious mechanical stimulation

(Figure 2A and 2B).

Because this noxious mechanical stimulation model seemed to activate a large proportion of the SPR-immunoreactive neurons in lamina I, the next test was to identify whether we could pharmacologically modulate the Fos activity in the SPR-immunoreactive neurons using the selective SPR anatagonist, CP-99994. CP-99994 is a potent, non-peptide antagonist selective for the neurokinin-1 receptor, i.e. the SPR, that is without the affinity for calcium channnels found in the related NK-1 antagonist, CP-96,345.

For intrathecal administration of CP-99994, intrathecal catheters were implanted in rats 7 days prior to testing, according to the methods described by Yaksh et al. On the day of testing, rats were maintained under a mixture of 2% halothane and 1% nitrous oxide anesthesia and received a 200 μg dose of CP-99994 intrathecally; control rats received the same dose of the inactive stereoisomer, CP-100,263. After a ten minute interval, the noxious mechanical stimulus was applied. Animals were perfused and tissue was processed as previously described above. Examination of the fos labeling in SPR-immunoreactive neurons of lamina I, in 25 μm sagittal section, showed that there was *no difference* in the number of double-labeled neurons found in rats that received the SPR antagonist versus rats that received the inactive isomer. In other words, the antagonist failed to block expression of the fos protein in SPR-immunoreactive neurons that were activated by noxious mechanical stimulation.

DISCUSSION

General observations on the distribution of the fos protein

Consistent with previous findings (Menetrey, et al., 1989; Morgan, et al., 1987; Morgan and Curran, 1991), we found that a subpopulation of spinal cord neurons express Fos like immunoreactivity in response to noxious stimulation of

different modalities. The stimulation protocols included a 50°C thermal stimulus, noxious pinch or plantar injection of 5% formalin, all delivered to the right hind paw. Regardless of the stimulus modality, the highest density of Fos like immunoreactive neurons was observed within the superficial laminae of the dorsal horn. This pattern of Fos like immunoreactive neurons directly correlates with the localization of nociresponsive neurons as well as the predominant termination of nociceptive primary afferents and thus appears to be restricted to spinal areas involved in nociceptive processing. However, because the factors involved in the induction of the Fos protein have not been completely delineated, we cannot be sure that the Fos like immunoreactive neurons that we observed are indeed nociceptive.

By comparison with previous studies on noxious evoked Fos like immunoreactivity, we found a sparse distribution of Fos like immunoreactive neurons in both the nucleus proprius (laminae III and IV), which contains mostly non-nociceptive neurons, and in the neck of the dorsal horn (laminae V and VI), which typically contains wide dynamic range neurons that respond to both noxious and non-noxious stimulation. This paucity of staining in the nucleus proprius of the dorsal horn is directly related to the fact the animals were maintained under constant anesthesia during the behavioral period. This minimizes non-nociceptive input transmitted by large diameter afferents, that occur in the awake, behaving animal. The differential pattern of labeling in the awake and anesthetized preparations support our contention that the pattern of labelling that we observed in the anesthetized animals is driven primarily by noxious stimuli.

Fos induction in substance P receptor -immunoreactive neurons

Double-labeling for the SP receptor and Fos protein revealed that subpopulations of SPR-immunoreactive neurons are indeed "activated" after noxious stimulation. Double labeled neurons were predominantly observed in lamina I, but also in the medial portions of laminae IV-V; all three nociceptive

models produced double-labeled neurons in these two areas.

Our observation that the small population of SPR-immunoreactive neurons located along the medial edge of laminae IV and V co-expressed a high level of Fos like immunoreactivity is significant. It suggests that these SPR-immunoreactive neurons are specifically "activated" by noxious stimulation, and that these SPR-immunoreactive neurons serve some function that is conserved in all modalities of noxious transmission.

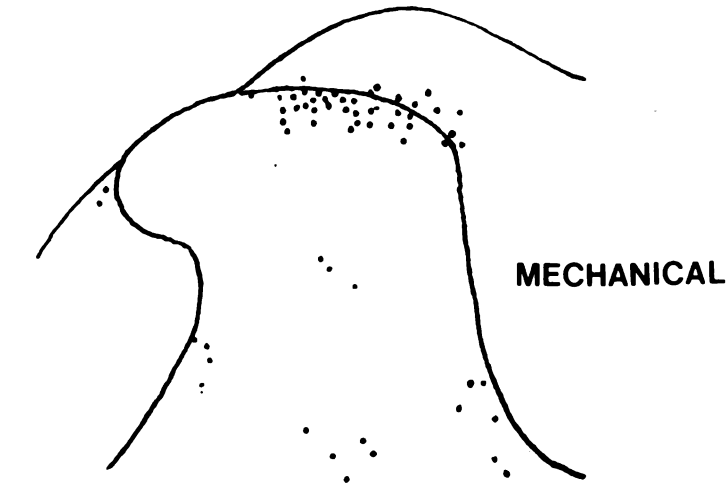
Although some information about the induction of fos in lamina I neurons could be discerned from transverse sections, closer examination in sagittal section showed that almost a third of SPR-immunoreactive neurons were double-labeled. In this case, the stimulation paradigm involved noxious mechanical stimulation. This finding confirms that a large proportion of marginal SPR-immunoreactive neurons are activated following noxious stimulation and suggests that these neurons are functionally important in nociceptive transmission. On the other hand, clearly the majority of neurons were not double labelled. Indeed although our results indicate that substance P is a factor that contributes to Fos induction by neurons in the superficial dorsal horn, SPR-immunoreactive neurons comprise less than 5% of the cells in lamina I, which emphasizes that many nociresponsive may not be activated by SP-containing primary afferent. The fact that the SPR antagonist, CP-99994, did not inhibit fos induction in these SPR-immunoreactive marginal neurons suggests activity in these neurons is mediated by convergence of SP and other neurotransmitters contained in nociceptive afferents. Indeed, the presence of a large proportion of Fos-like immunoreactive neurons that did not express the SPR suggests that many other factors besides SP are involved in the induction of Fos (and the transmission of nociceptive messages by dorsal horn neurons). It is of interest in this regard that our preliminary studies showing that intrathecal administration of SP is not sufficient to evoke Fos in dorsal horn neurons is

consistent with this hypothesis.

On the other hand, other reports have shown that intrathecally administered SPR antagonists partially inhibit the nociceptive behaviors evoked following subplantar injection of formalin (Chapman and Dickenson, 1993; Yamamoto and Yaksh, 1992). Moreover, NK-1 antagonists have been shown to selectively inhibit pain-like behaviors provoked by intrathecal injection of SP (Xu et al., 1992; Yashpal et al., 1993). Finally, Cutrer et al. (Cutrer et al., 1995) reported that systemic injection of NK-1 antagonists decreases fos expression after noxious chemical stimulation and Hill and colleagues (personal communication) found that high doses of CP-99994 reduced the expression of Fos in the trigeminal nucleus caudalis that is produced by electrical sitmulation of the trigeminal nerve.

It is difficult to account for the fact that others have found inhibitory effects of NK-1 antagonists on noxious stimulus evoked fos expression, whereas we did not. Several possible explanations can be offered. First, we used anesthetized preparations; this may have sufficiently reduced overall Fos expression to a level that could not be further inhibited by NK-1 antagonists. Second the fact that even very high intrathecal doses only partly block the pain associated with the formalin test suggests that intrathecal injection of NK-1 antagonists is not the ideal route. This unfortunately is complicated both by the fact that the best NK-1 antagonists for the rat receptor poorly cross the blood brain barrier while the one that crosses the blood brain barrier, namely CP-99994, has greatest affinity for the guinea pig rather than the rat NK-1 receptor (Watling and Krause, 1993). Despite these concerns and in light of the possibility that systemic injection is the best route, we have repeated our analysis, specifically addressing the effect of systemic administration of an NK-1 antagonist on the induction of Fos by intense noxious chemical stimulation in the awake, behaving rat. The next chapter describes these studies.

Figure 1. These schematics illustrate the distribution of fos- like immunoreactive neurons in the lumbar spinal cord of rats that received one of three noxious stimulus modalities. All forms of noxious stimulation evoked similar distributions of fos-like immunoreactive neurons. The largest population of labelled neurons was found in the medial part of the superficial laminae (I and IIo). The most medial part of the neck of the dorsal horn and the more lateral reticulated region of lamina V contained additional labelled neurons, as did the area of the lateral spinal nucleus. A: mechanical noxious stimulus B: thermal noxious stimulus C: chemical noxious stimulus.



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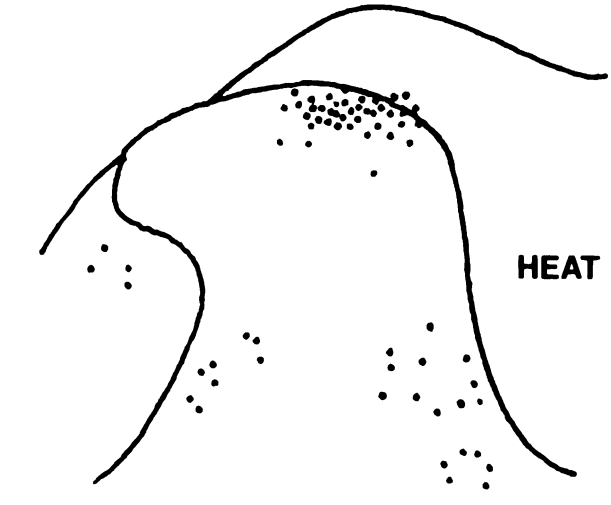

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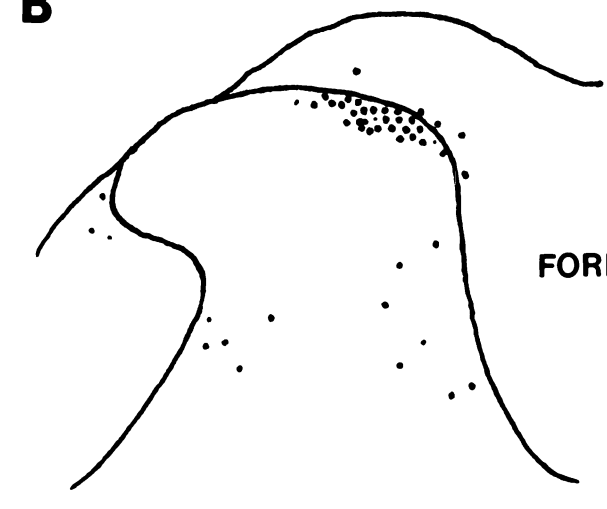


C

Figure 2. These sagittal sections through the lumbar spinal cord illustrate SPR-immunoreactive neurons (brown reaction product) in lamina I that are double-labeled (arrowheads) for Fos (pink nuclear reaction product) after noxious mechanical stimulation. Fos like immunoreactive neurons that are not double-labeled for SPR can also be seen. Calibration bar equals 50 μm.

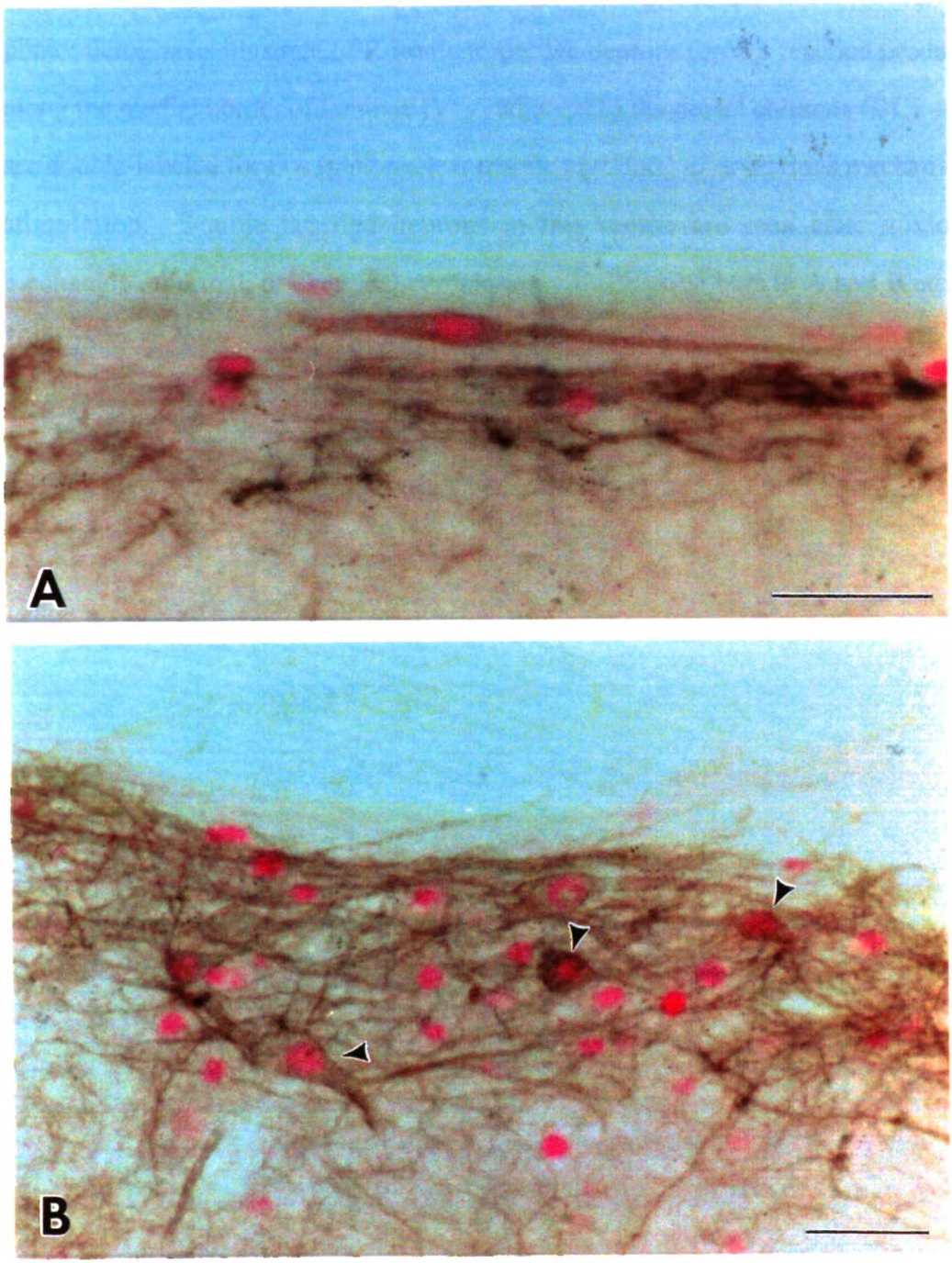
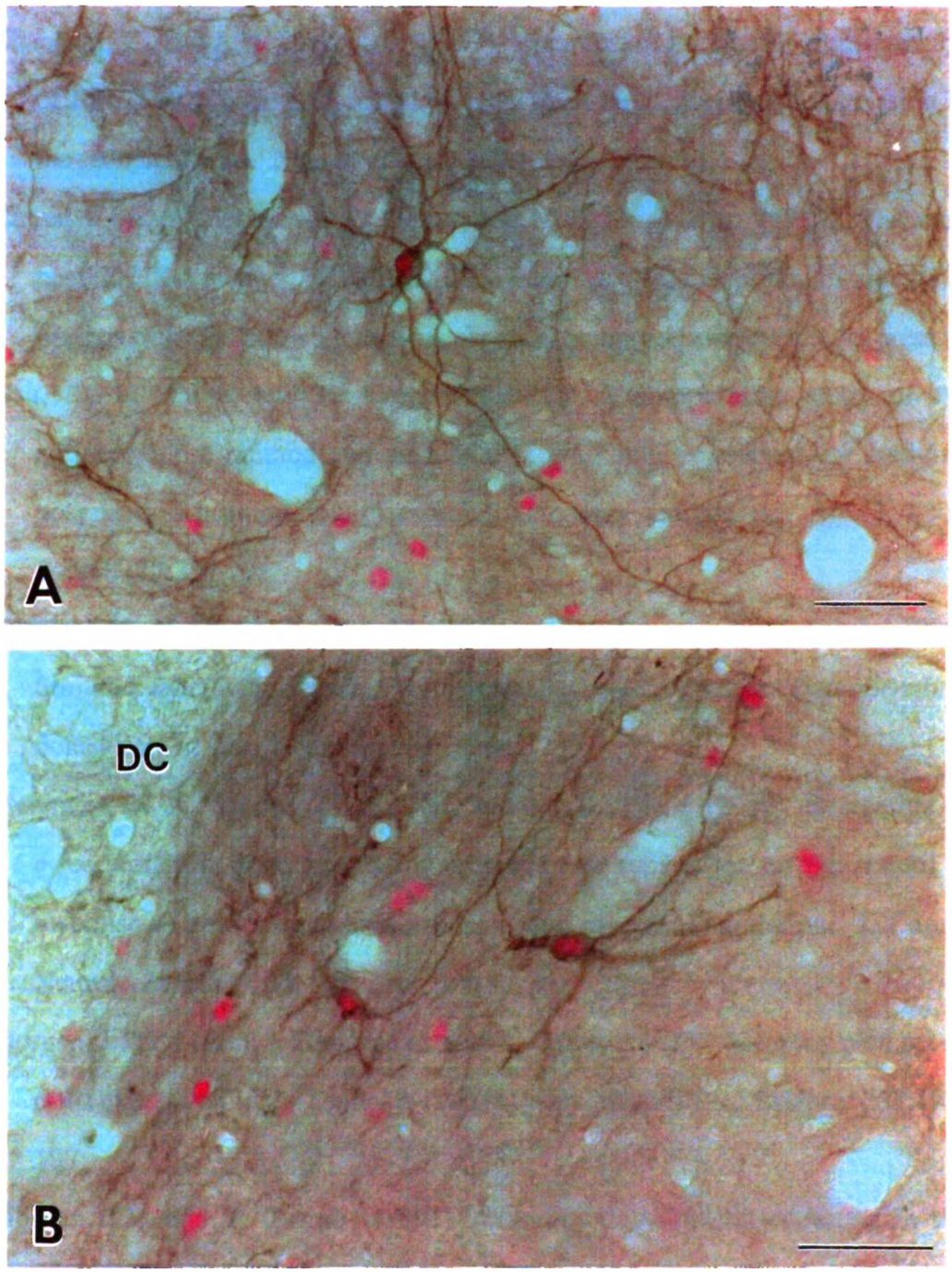

A
B

Figure 3. These transverse sections through the lumbar spinal cord illustrate photomicrographs illustrate SPR-immunoreactive neurons (brown reaction product) along the medial border of laminae IV-V, adjacent to the dorsal columns (DC), that are double-labeled for Fos (pink nuclear reaction product) after noxious mechanical stimulation. Double labelled neurons in this region are seen after noxious mechanical, thermal, or chemical stimulation. Calibration bars in A and B equal 100 μm.


A
DC
B

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CHAPTER VII

The non-peptide SPR antagonist, CP-99994, decreases c-fos expression produced by noxious chemical stimulation

INTRODUCTION

The neuropeptide substance P (SP) makes an important contribution to the processing of nociceptive signals in the dorsal horn of the spinal cord. Spinal cord substance P is largely derived from the small diameter unmyelinated primary afferent C-fibers, many of which respond to noxious stimulation. The SP containing C-fibers terminate predominantly in the superficial laminae (I and IIo), which contain nociresponsive neurons (Barber et al., 1979; Hokfelt et al., 1975; Ljungdahl et al., 1978). Electrophysiological recordings of spinal cord neurons show that nociresponsive neurons are selectively excited by iontophoresis of SP; non-nociceptive neurons do not respond (Salter and Henry, 1991); however see Fleetwood et al (1990 and 1993). Other studies demonstrated that SP can increase excitability of dorsal horn neurons through the induction of a prolonged depolarization. This appears to be generated in part by the enhancement of effects mediated through the NMDA receptor channel (Randic et al., 1990; Rusin et al., 1992). Finally, intrathecal injection of substance P elicits a stereotypic behavior that includes scratching and biting at the limbs and vocalization. Whether or not these behaviors are representative of "pain" behavior is uncertain (Cridland and Henry, 1988; Hylden and Wilcox, 1981).

Numerous studies have shown that noxious stimulation evokes the release of substance P in the spinal cord. For example, electrical stimulation of peripheral nerve at C-fiber intensity evokes the release of SP in the CSF and noxious mechanical or thermal stimulation increases the release of SP in the spinal cord dorsal horn (Duggan et al., 1988; Kuraishi et al., 1989). Since the intensity of the stimuli in these latter studies was often associated with inflammation, it is possible that C fiber inputs that are produced during the course of inflammation are the

critical factor in evoking substance P release. For this reason, in the present study we have elected to use mustard oil injection as the noxious stimulus. Mustard oil evokes a very selective and sustained activation of the C-fiber afferents and also produces a large inflammatory response in the injected limb (Heapy et al., 1987; Woolf and King, 1990; Woolf and Wall, 1986). Use of this very specific stimulus will allow us to discipher the contribution of SP to the activation of dorsal horn neurons.

To identify the postsynaptic targets of primary afferent derived SP, we have characterized the populations of spinal cord neurons that express the substance P receptor (SPR)/neurokinin-1 receptor (NK-1), using an antibody directed against the SPR. We found the SPR to be expressed at high densities over almost the entire somatic and dendritic surface of subpopulations of neurons, such that immunocytochemical detection of the receptor resulted in a Golgi-like filling of neurons that allowed morphological characterization of SPR immunoreactive neurons. The highest density of SPR-immunoreactive neurons in the spinal cord is in lamina I, a region that also contains very high concentrations of primary afferent derived SP. SPR-immunoreactive marginal neurons typically were fusiform, with long dendrites directed rostrocaudally. Conversely, the substantia gelatinosa (SG) was largely devoid of SPR-immunoreactive neurons, despite the heavy input of primary afferent SP into lamina II. A much smaller population of SPR-immunoreactive neurons was identified throughout laminae III-VI; these neurons were typically large and multipolar neurons with dendrites that extended dorsally towards and often into the superficial laminae.

In a previous report, we examined the level of "activity" of the SPR-immunoreactive neurons by monitoring noxious stimulus evoked induction of the c-fos proto-oncogene in SPR-immunoreactive neurons. We found that subpopulations of SPR immunoreactive neurons are selectively double-labeled for

the Fos protein following noxious stimulation. In the present study we have extended those observations, by evaluating the effect of SP antagonists on the induction of Fos in SPR-immunoreactive neurons. In order to maximally activate the neurons, we used mustard oil injections, a noxious stimulus that selectively activates C-fibers.

METHODS

Materials:

Mustard oil (allyl isothiocyanate; Sigma) 20 mg was diluted in 1.0 ml mineral oil and stored at 4°C. The selective SPR antagonist, CP-99994 and its inactive stereoisomer, CP-100, 263 were both obtained from Merck, Sharp and Dohme, UK. CP-99994 and CP-100,263 were dissolved in 0.9% saline just prior to use.

Protocol:

Male Sprague-Dawley rats (350-400 g; Bantin and Kingman) were used in this study. For i.v. drug infusions, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (7.0 mg/kg), and polyethylene catheters (PE-10) catheters were secured into the jugular vein and externalized . After surgery, the rats were allowed to recover for 2 days prior to beginning the experimental protocol. On the day of testing, the rats were anesthetized with Halothane for a brief period (approximately two minutes) so that we could make an i.v. injection of either CP-99994 or CP-100-263, without producing excess agitation and movement that might interfere with the pattern of Fos expression in the lumbar spinal cord.

The drugs were dissolved in a 50 µl of saline, which was injected over a period of 30 seconds; this was followed by a saline flush of 50 µl. After the i.v. injection, the rats were placed in a plexiglass chamber so that their behavior could be observed. Ten minutes later, the rats were removed from the chamber, and then

gently restrained so that we could make a plantar injection of mustard oil (20%, 50 µl) into the right hindpaw. Immediately after, the rat was replaced into the behavioral chamber, for one hour. The behavior produced by mustard oil injection included some licking and flinching of the injected paw, however, the intensity of the behavior was less than that produced by formalin injection. There was no vocalization and very little movement.

Tissue Processing:

After one hour, the rats were reanesthetized with ketamine (60 mg/kg) and xylazine (6.0 mg/kg) and intracardially perfused with 50 ml 0.05 M phosphate buffered saline (PBS) pH7.4 at 37°C followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The spinal cords were removed and postfixed for 5 hours in the same fixative. The tissues were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer for two days at 4°C. Using a freezing microtome, spinal cords were sectioned either at 50 µm transverse sections or 25 µm sagittal sections. To evaluate the overall effect of the antagonists on Fos expression, the sections were cut transversely. To evaluate the effect on SPR-immunoreactive in the superficial dorsal horn we examined sections cut in the sagittal plane.

For double labeling of the Fos protein and the substance P receptor, we used sequential avidin-biotin procedures (Hsu et al., 1981). The SPR is first visualized with a protocol that produces a black-brown reaction product; this is followed by the Fos protocol using a chromogen that produces a red reaction product. In brief, spinal cord sections were incubated overnight at room temperature in a rabbit polyclonal primary antiserum directed against the C-terminal portion of the substance P receptor (1:20,000; courtesy of Stephen Vigna, Duke University). After incubation in the primary antiserum, sections were sequentially incubated in biotinylated goat anti-rabbit IgG avidin-biotin-peroxidase complex

(Vector Laboratories, Burlingame, CA). The reaction product was visualized using hydrogen peroxide and diaminobenzidine (DAB) as the chromogen with nickel intensification. Following SPR immunocytochemistry, a similar process was repeated to visualize the Fos protein. In this case, the SPR reacted tissue is incubated with a polyclonal antiserum directed against an *in vitro* translated protein product of the c-fos gene (1:20000: courtesy of Dennis Slamon, Department of Hematology and Oncology, UCLA). After incubation in the primary Fos antibody, the sections were incubated in biotinylated goat anti-rabbit IgG avidin-biotin-phosphatase complex (Vector Laboratories, Burlingame, CA). The Fos protein was visualized as a bright red reaction product. After the second staining, the sections were mounted on gelatin coated Superfrost slides, which were later dehydrated and coverslipped. Double labeled neurons were outlined with the brown/black SPR immunoreaction product and contained pink nuclei.

To quantify the double labeling, we counted the total number of SPR-immunoreactive neurons and compared this to the number of Fos-immunoreactive SPR-immunoreactive (i.e. double labelled neurons). The results are given in percentages of double-labeled neurons.

RESULTS

Fos expression

Subcutaneous injection of mustard oil in the hind paw produced a very distinct pattern of fos-like immunoreactive (FLI) neurons in the lumbar enlargement of the spinal cord. Labelled neurons were predominantly concentrated on the side ipsilateral to the site of mustard oil injection; a few were recorded on the contralateral side. The majority of FLI was expressed by neurons in the medial half of lamina I and lamina IIo of the spinal cord (120 cells per section), which corresponds to the topographic location of the sciatic nerve input. The nucleus proprius was essentially devoid of FLI neurons. The neck of the dorsal horn,

particularly in the midportion of lamina V, contained approximately 25 neurons per 50 μm section. Bordering the central canal, we recorded another small group of FLI neurons; these were concentrated along the medial aspects of laminae VI and VII.

The rostral caudal distribution of FLI neurons was somewhat more limited compared to that observed with other inflammmatory chemical stimuli. Thus, the densest concentration of FLI neurons was confined to the superficial laminae of lumbar segments 4 and 5, which corresponds to the location of termination of afferents from the hind paw. Many fewer cells were recorded immediately rostral and caudal to L4.

Drug Treatment

Pretreatment with the substance P receptor antagonist CP-99994 significantly reduced the number of FLI neurons in the superficial dorsal horn of the lumbar enlargement ipsilateral to the hindpaw that received the injection of mustard oil. The number of FLI neurons in the superficial laminae decreased by approximately 35% after treatment with 1.0 mg/kg i.v. CP-99994 (ANOVA, $p<.01$; Figure 1). On the other hand, CP-99994 was without significant effect on the numbers of Fos-immunoreactive neurons in the deeper laminae of the cord. In animals pretreated with the inactive enantiomer CP-100,263 we found no inhibition of Fos expression compared to saline injected controls.

Substance P receptor-immunoreactive neurons that coexpress the Fos protein

The percentage of SPR-immunoreactive neurons in the superficial dorsal horn that colocalized the Fos protein was higher than we observed with the other stimuli; thus in the medial half of lamina I approximately 60% of the SPR neurons were double-labeled for the Fos-like immunoreactivity after mustard oil injection (Figure 2). Despite the significant decrease in the overall number of fos-

immunoreactive neurons, we found that the SPR antagonist, CP-99994, did not significantly change the percentage of SPR-immunoreactive neurons that expressed the Fos protein in lamina I.

DISCUSSION

The distribution of Fos-like immunoreactive neurons produced by mustard oil injection in the hind paw was more restricted than what we observed with other noxious stimuli. Specifically, the labelling was concentrated in the lumbar enlargement (L4 and 5). Compared to the pattern of labeling produced by formalin injection, we found a more restricted rostrocaudal distribution, and significantly less labeling in the nucleus proprius. Presumably the latter reflects the fact that there is much less shaking of the paw and less movement overall after mustard oil injection than after formalin injection. We believe that the more restricted pattern of labelling reflects the fact that mustard oil is a more selective activator of C-fibers. Indeed the pattern of labelling produced by mustard oil is more similar to that seen after capsaicin injection (unpublished observations). Formalin may simultaneously activate a larger range of peripheral afferent fibers.

The 35% reduction in Fos expression in laminae I and IIo suggests that SP does contribute to the transmission of nociceptive messages in dorsal horn neurons, and that an antinociceptive effect can be produced by NK-1 antagonists. This result is consistent with a recent unpublished study that demonstrated a significant reduction of Fos evoked by intracisternal injection of capsaicin by the NK-1 antagonist RPR 100,893 (Cutrer et al., 1995). However, our results are not in agreement with the electrophysiological findings of Munro et al. (Munro et al., 1993), which reported that NK-2, but not NK-1 antagonists inhibit mustard oil evoked firing of dorsal horn multireceptive neurons. The fact that Munro et al. only tested neurons in laminae IV-V may account for the disparity in results; recordings

might have missed an NK-1 mediated nociceptive transmission in the superficial dorsal horn. The fact that we found considerable numbers of SPR-immunoreactive double labeled neurons in the superficial dorsal horn supports that hypothesis. Another point is that those authors used peptide antagonists, which are far less selective than the non-peptide antagonists. Thus, those studies may have missed an NK-1 contribution to the activation of nociresponsive neurons neurons in the superficial laminae.

Although the presence of many SPR immunoreactive neurons that were double labelled for the Fos protein is consistent with the hypothesis, the fact that the SPR/NK-1 antagonist CP-99994 had no effect on the number of double-labeled marginal neurons indicates that SP is not a necessary contributor to the activation of Fos in these neurons. It is of interest in this regard that intrathecal injection of SP, although evoking clear behavioral signs, does not induce significant increases in Fos like immunoreactivity. The most likely explanation is that co-released neurotransmitters (notably glutamate, acting via both AMPA and NMDA receptors) contribute to the Fos induction in neurons that express the SPR. It is possible that there was a reduction in the intensity of staining; this would be difficult to detect using immunostaining procedures.

More puzzling is the overall reduction of 35% in the expression of Fos immunoreactivity, in neurons that did not express the SPR. We suggest that this resulted from a generalized lowering of activity in dorsal horn neurons in response to the NK-1 antagonists. For example, if the activity of neurons that express the SPR were sufficiently reduced by the NK-1 antagonists, and if these are a necessary contributor to the activity of other neurons in the superficial dorsal horn (i.e. they contribute to the excitability of the overall population), it is possible that they would no longer express Fos in response to mustard oil injection. This hypothesis presupposes that those neurons that express the SPR are upstream of

many of the other neurons in the superficial dorsal horn. It is significant that only about 5% of the marginal neurons express the SPR. For SP and for this population of neurons to be significant to the transmission of nociceptive messages, it is reasonable to suggest that they are critical to the activation of other marginal neurons.

Although the SPR antagonists did not significantly alter the expression of Fos immunoreactivity in the neurons expressing the substance P receptor, there was a qualitative difference noted in the staining of CP-100,263 treated tissue versus CP-99994 treated tissue. The pattern of SPR immunoreactivity in the normal, untreated animal shows SPR immunoreactivity densely concentrated along the surface of the soma and dendrites of labelled neurons. When mustard oil was administered in the presence of the inactive isomer, CP-100,263 we detected a change in this pattern; SPR immunoreactivity was now clustered into small patches within the cell soma. Based on related *in vivo* studies in our laboratory, and consistent with other *in vitro* studies, we believe that this redistribution of receptor reflects internalization of the receptor by endocytosis. In the CP-99994 treated tissue, mustard oil injection was less effective in producing internalization of the SPR; the distribution of the SPR was more similar to that of a normal, untreated animal (Figure 3). This provides some evidence that the antagonist was, in fact, counteracting an effect which is generated via SP.

In summary, this study has shown that hindpaw injection of the noxious chemical, mustard oil, which selectively activates C-fibers, evokes a distinct distribution of Fos-like immunoreactivity in the superficial laminae of the spinal cord, which mirrors the terminations of C-fiber derived SP. Furthermore, we found that while an SPR antagonist significantly reduced mustard oil evoked Fos expression, we could not block Fos expression in SPR-immunoreactive neurons. Whether Fos expression was reduced could not be discerned. These findings

indicate that multiple neurotransmitters contribute to mustard oil induced activity of nociresponsive neurons in the superficial dorsal horn.

Figure 1. These photomicrographs illustrate the pattern of mustard oil evoked Fos-like immunoreactivity in the dorsal horn (dh) of the lumbar enlargement of rats that received the inactive isomer (A) CP-100,263 and the NK-1 anatgonist (C) CP-99994. (B) and (D) are higher magnifications of the superficial laminae in photomicrographs of (A) and (C), showing the difference in density of Fos-like immunoreactive neurons in the superficial laminae. Calibration bars equal 300μm in A and C; 100μm in B and D.

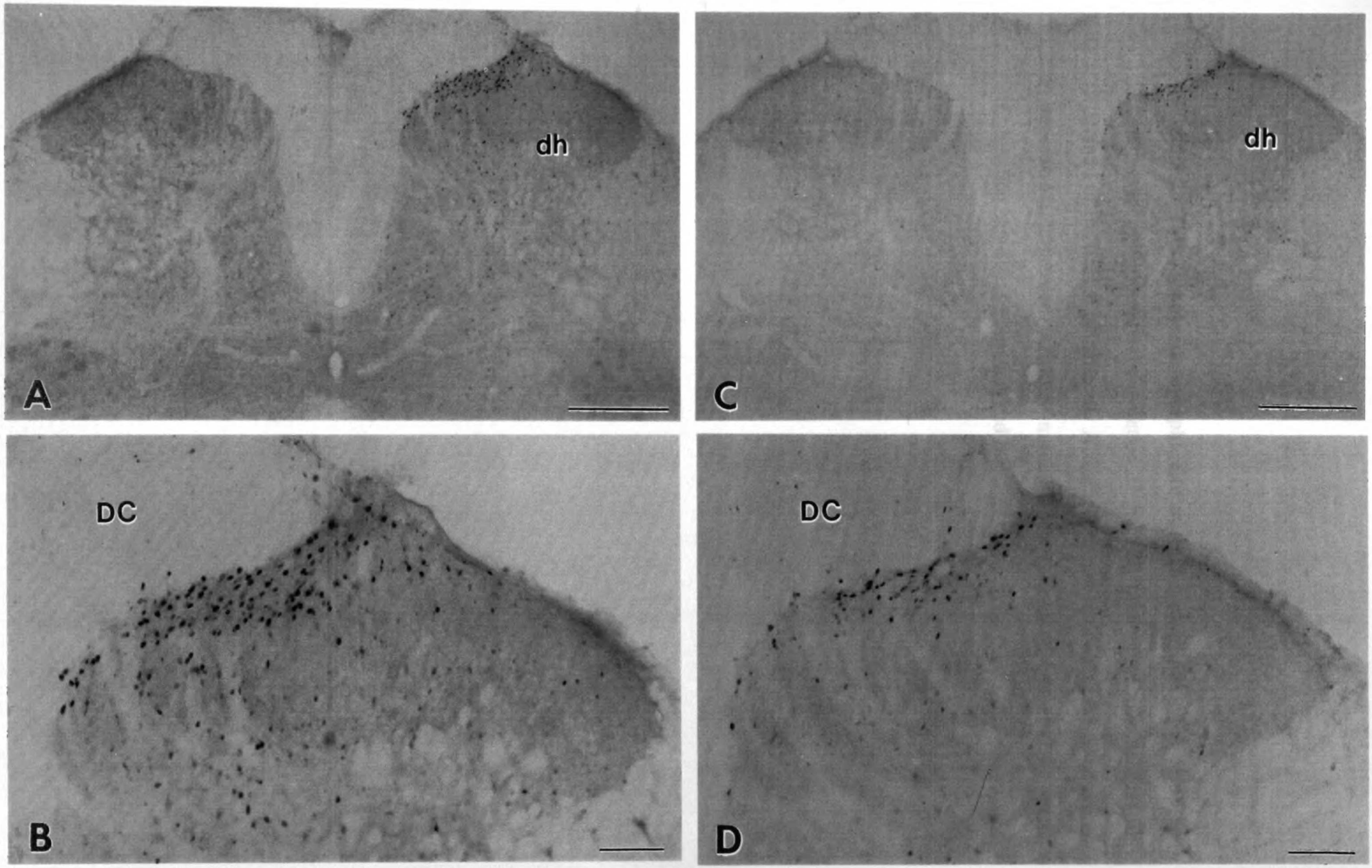
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Figure 2. These photomicrographs illustrate the distribution of Fos-like immunoreactivity in the subpopulation of the SPR immunoreactive neurons in lamina I of the lumbar spinal cord. Arrowheads point to double-labeled neurons; several single-labeled Fos-like immunoreactive neurons can also be seen. Calibration bars equal 50μm in A and B.

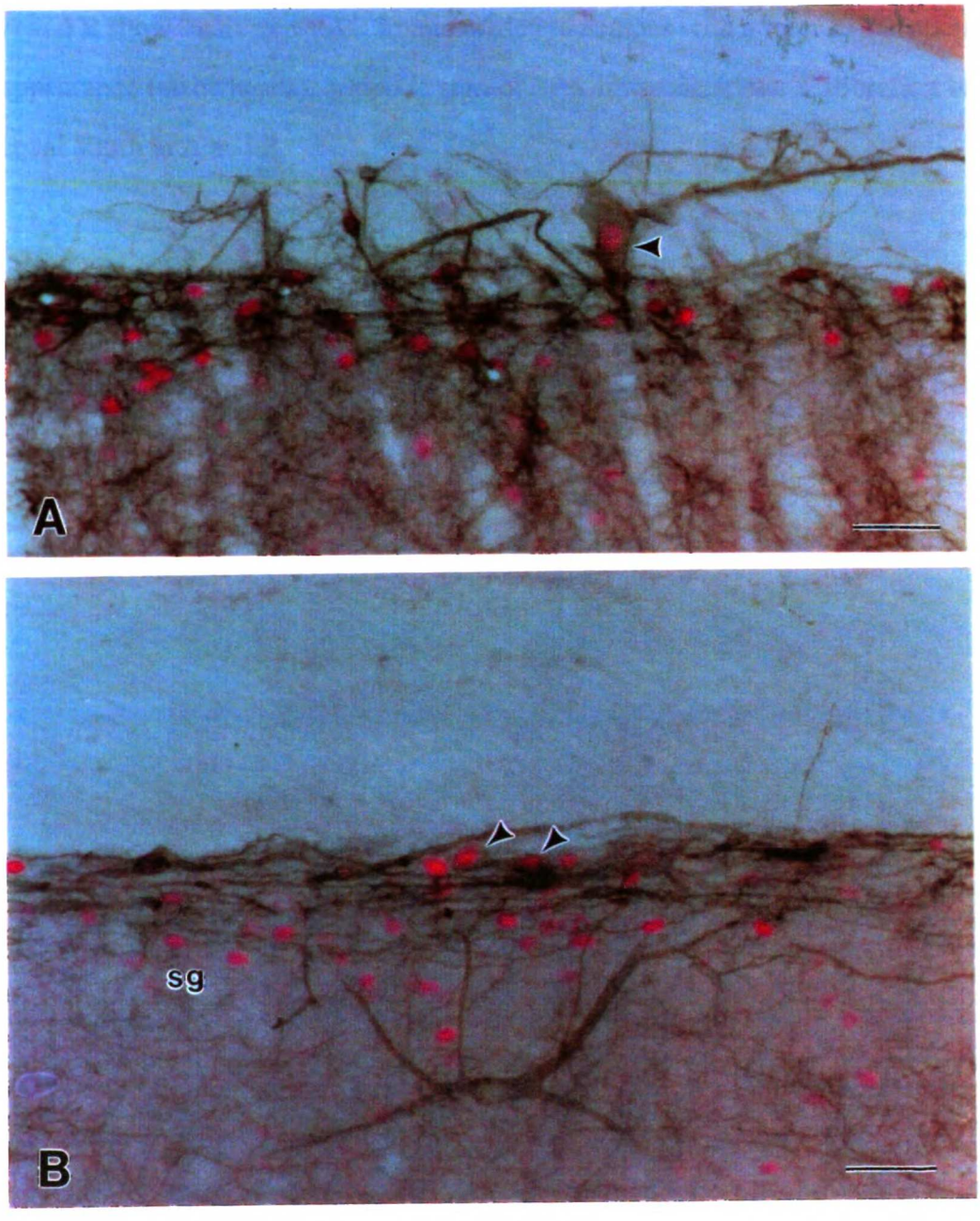
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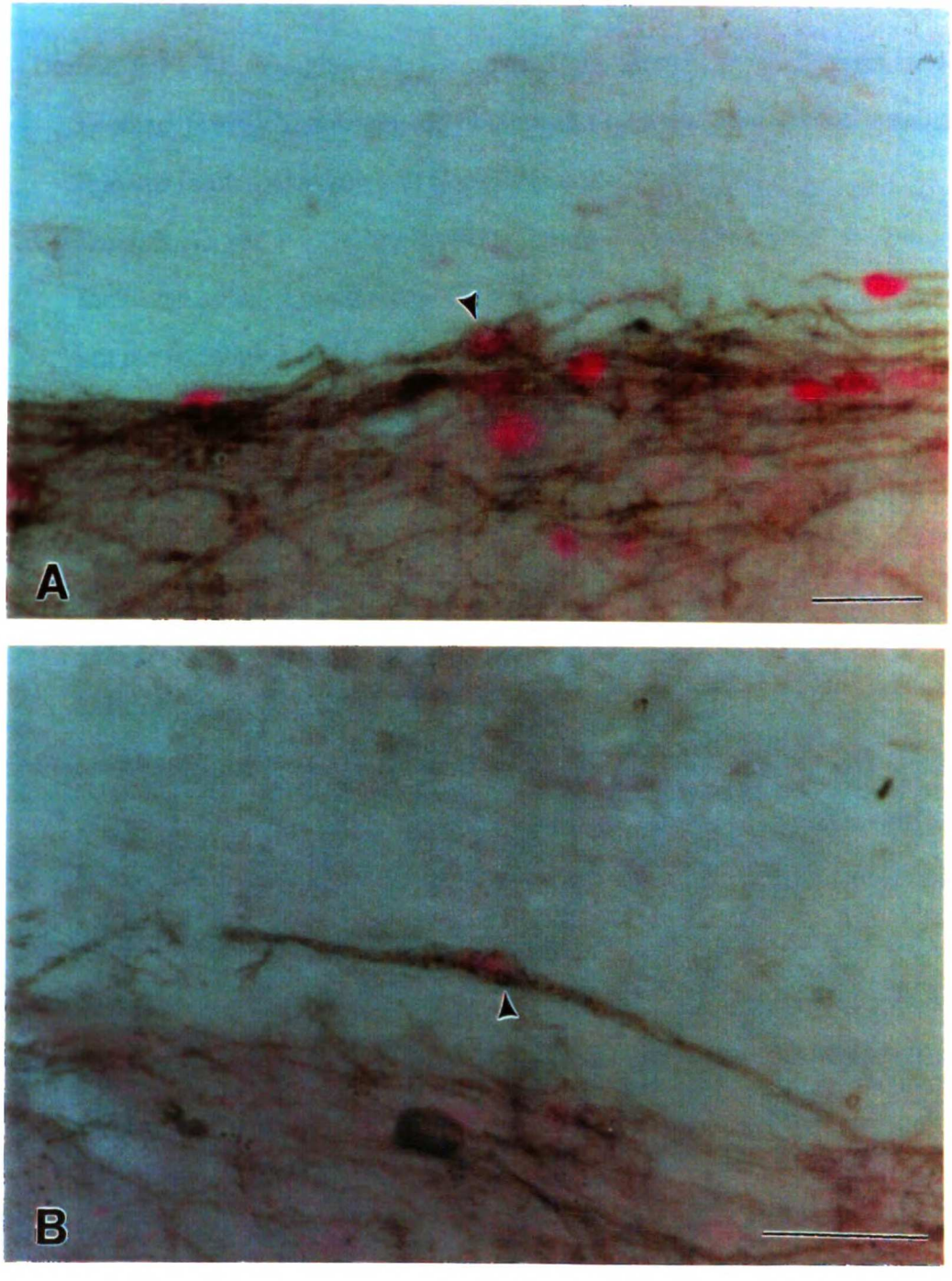
Figure 3. These photomicrographs illustrate SPR internalization observed in SPR immunoreactive neurons that also show Fos activation after mustard oil injection. A and B show "activated" SPR immunoreactive neurons with a mottled and spotted appearance (arrowheads), which is sign of SPR internalization. Calibration bars equal 50μm in A and B.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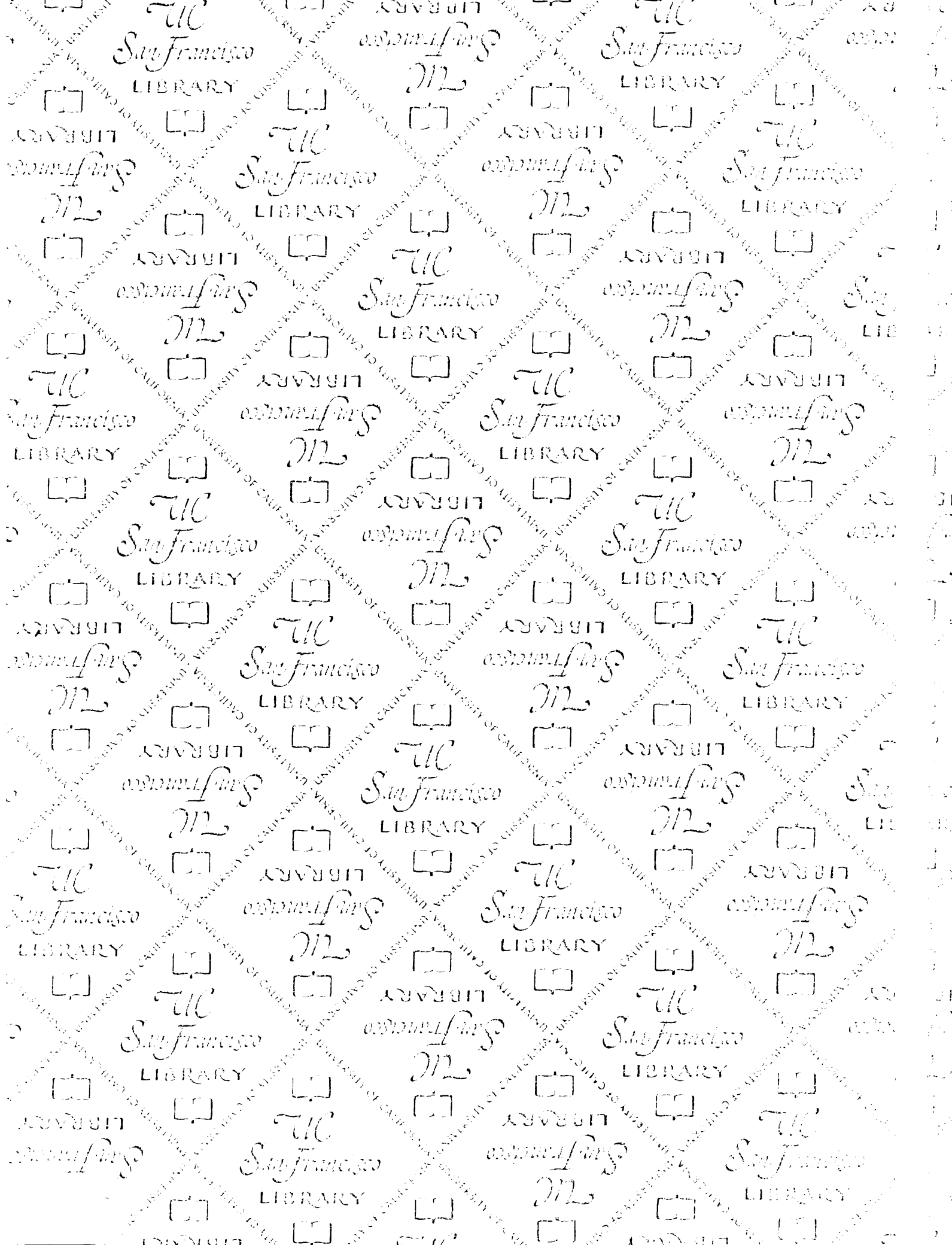
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