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Preferential synaptic relationships between substance P-immunoreactive boutons and neurokinin 1 receptor sites in the rat spinal cord

(receptor mismatch/dorsal horn/tachykinin)

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ABSTRACT **Substance P plays an important role in the transmission of pain-related information in the dorsal horn of the spinal cord. Recent immunocytochemical studies have shown a mismatch between the distribution of substance P and its receptor in the superficial laminae of the dorsal horn. Because such a mismatch was not observed by using classical radioligand binding studies, we decided to investigate further the issue of the relationship between substance P and its receptor by using an antibody raised against a portion of the carboxyl terminal of the neurokinin 1 receptor and a bispecific monoclonal antibodies against substance P and horseradish peroxidase. Light microscopy revealed a good correlation between the distributions of substance P and the neurokinin 1 receptor, both being localized with highest densities in lamina I and outer lamina II of the spinal dorsal horn. An ultrastructural double-labeling study, combining preembedding immunogold with enzyme-based immunocytochemistry, showed that most neurokinin 1 receptor immunoreactive dendrites were apposed by substance P containing boutons. A detailed quantitative analysis revealed that neurokinin 1 receptor immunoreactive dendrites received more appositions and synapses from substance P immunoreactive terminals than those not expressing the neurokinin 1 receptor. Such preferential innervation by substance P occurred in all superficial dorsal horn laminae even though neurokinin 1 receptor immunoreactive dendrites were a minority of the total number of dendritic profiles in the above laminae. These results suggest that, contrary to the belief that neuropeptides act in a diffuse manner at a considerable distance from their sites of release, substance P should act on profiles expressing the neurokinin 1 receptor at a short distance from its site of release.**

Substance P (SP) is a neuropeptide prominently expressed in small sensory primary afferents that is involved in the modulation of pain-related information in the spinal dorsal horn (1, 2). The central terminations of SP-containing primary sensory afferents occur mainly in laminae I and II (3, 4). There is abundant evidence supporting the notion that SP acts preferentially on the neurokinin 1 receptor (NK-1r) (5). The distribution of the NK-1r in the spinal cord has been studied extensively by using radioactive ligand binding and, more recently, immunocytochemistry. The former approach has shown a close match between the distribution of SP immunoreactivity and NK-1r binding sites in the superficial laminae of the dorsal horn (6, 7). In contrast, studies using two different antibodies against the NK-1r have shown a conspicuous lack of NK-1r immunostaining in lamina II (8–10), an area that is abundantly innervated by SP. Therefore, these results using immunocytochemistry do not favor a direct synaptic association between SP-containing afferents and neurons expressing the NK-1r. A confocal microscopic study adds to the controversy by showing that NK-1r immunoreactive neurons with cell bodies in laminae III-IV received a substantially higher number of appositions from SP immunoreactive boutons than neurons that expressed choline acetyltransferase but that were not NK-1r immunoreactive (11). This latter observation, together with our own results showing a preferential innervation by SP of nociceptive neurons (12), has led us to believe that SP in the spinal cord might not act in a diffuse manner as had been suggested (8) but, rather, that it might activate the NK-1r at a short distance from its site of release. To address this unresolved issue, we used a new and well characterized anti-NK-1r antibody in a stringent quantitative study at the ultrastructural level. In this study, a double-labeling approach was used in an attempt to define the relationship of NK-1r containing dendritic profiles in relation to its SP input in laminae I-III of the spinal dorsal horn.

MATERIALS AND METHODS

SP and NK-1r Immunostaining for Light Microscopy. Four male Wistar rats (275–350 g) were anesthetized with Equithesin and were perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cord segments C3-C5 were removed and postfixed in the same fixative for 2 hours at 4°C and then were infiltrated in 30% sucrose in 0.1 M PB. Transverse serial sections (50 μm thick) were obtained with a freezing sledge microtome and were collected in PBS with 0.2% Triton X-100 (PBS+T). The sections were processed for the demonstration of either SP or NK-1r immunoreactivity in serial sections.

For SP immunostaining, the sections were incubated with a bispecific anti-SP/anti-horseradish peroxidase monoclonal antibodies (1:10; code P4C1) (13) overnight at 4°C. The sections subsequently were incubated in 5 μg/ml of Sigma type VI horseradish peroxidase in PBS+T for 1 hour. After extensive washing in PBS+T, the sections were reacted with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in PBS+T.

For NK-1r immunostaining, an immune-affinity purified rabbit anti-NK-1r antiserum was used for all studies and was prepared as described (14). The specificity of the anti-NK-1r

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Abbreviations: IR, immunoreactive; NK-1r, neurokinin-1 receptor; PB, phosphate buffer; SP, substance P; PBS+T, PBS with 0.2% Triton X-100.

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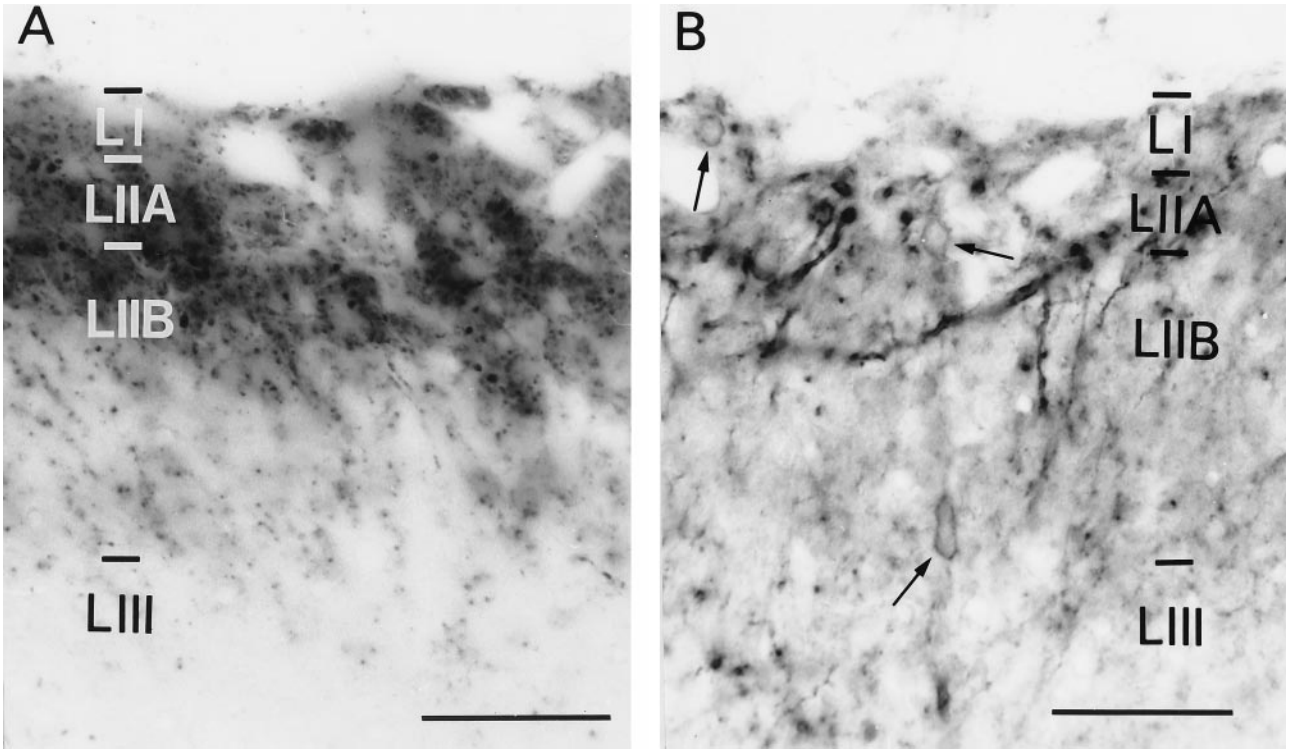

FIG. 1. Light microscopic distribution of SP and NK-1r immunoreactivity in serial sections of the rat spinal dorsal horn. In *A*, SP immunoreactivity was distributed in all superficial laminae with the highest density in lamina I (LI) and outer lamina II (LIIA) and the lowest in inner lamina II (LIIB) and lamina III (LIII). SP immunoreactivity was associated with axonal fibers and varicosities. In *B*, the distribution of NK-1r immunoreactivity was similar to that of SP, being distributed in all superficial laminae with highest densities in LI and LIIA and lowest in LIIB and LIII. NK-1r immunoreactivity was associated with dendrites and cell bodies (arrows). (Bars = 50 μm.)

antibody has been established by using ELISA, immunoblots, immunocytochemical labeling of transfected cells, and immunohistochemical experiments in postnatal rat as well as in postnatal and adult mouse forebrain sections (14). The sections were pretreated with 5% normal goat serum (Sigma) in PBS+T for 30 minutes followed by incubation with the NK-1r antiserum (1:25) for 48 hours at 4°C. The sections then were incubated with a goat anti-rabbit biotinylated IgG (1:3,000; ICN) for 2 hours followed by incubation with an ABC complex (1:50) from an Elite ABC kit (Vector Laboratories) for 1 hour at room temperature. Subsequently, the sections were reacted with 3,3′-diaminobenzidine tetrahydrochloride in PBS+T. Between incubations, sections were washed extensively in PBS+T. The free-floating sections were mounted onto gelatin-subbed slides, were dehydrated in ascending alcohols, were cleared in xylene, and were coverslipped with Entellan (Merck).

SP and NK-1r Double-Labeling for Electron Microscopy. Four male Wistar rats (275–350 g) were anesthetized with Equithesin and were perfused through the ascending aorta with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PB for 30 minutes, followed by perfusion with 4% paraformaldehyde, and finally with 10% sucrose in 0.1 M PB (30 minutes each). Spinal cord segments C3-C5 were removed and infiltrated in 30% sucrose in 0.1 M PB, were snap frozen by immersion in liquid nitrogen, and were thawed in 0.1 M PB at room temperature. Transverse sections (50 μm thick) were obtained with a Vibratome and were treated with 1% sodium borohydride in 0.01 M PBS for 30 minutes. After extensive washings with PBS until all of the bubbles disappeared (15), the sections were pretreated with 0.5% BSA in PBS for 30 minutes.

For double-labeling of SP and the NK-1r, SP was detected by using an immunoperoxidase method, and the NK-1r was detected by using the preembedding immunogold–silver method. Triton X-100 was omitted from all of the steps. The sections were incubated in a mixture of P4C1 antibody (1:10) and rabbit anti-NK-1r antiserum (1:10) in 0.1% BSA in PBS for 48 hours at 4°C. SP immunostaining was performed as for light microscopy. The 3,3′-diaminobenzidine tetrahydrochloride reaction was carried out, however, with double intensification (16). For NK-1r immunostaining, the sections were rinsed with PBS and were incubated in a solution containing 0.1% gelatin and 0.8% BSA for 10 minutes. Sections then were incubated with a goat anti-rabbit-IgG coupled to 1-nm gold particles (1:25; Biocell Laboratories) overnight at 4°C. The sections were rinsed with the solution of 0.1% gelatin and 0.8% BSA and then were rinsed with PBS. Sections subsequently were incubated in 2% glutaraldehyde in PBS for 10 minutes followed by extensive washings with PBS and 0.2 M sodium citrate buffer. Silver intensification of the gold particles was performed for 15 minutes by using a silver enhancement kit (Amersham). The sections were washed in the sodium citrate buffer followed by 0.1 M PB and then were incubated in 1% osmium tetroxide in 0.1 M PB for 1 hour at room temperature. Finally, the sections were dehydrated in ascending alcohol concentrations and propylene oxide and were flat embedded in Epon as described (17). After polymerization of the Epon, the sections were examined by light microscopy, and the selected fields were re-embedded and trimmed for electron microscopic examination. The ultrathin sections were collected on formvar-coated one-slot grids, were counterstained with uranyl acetate and lead citrate, and were observed with an electron microscope (Philips 410, Philips Electron Optics Canada, Scarborough, ON, Canada).

Quantitative Analysis. Four ultrathin sections, each corresponding to a different Epon block, were quantified for each of the four animals. Four random fields in each of laminae I, IIA, IIB, and III were photographed at a magnification of 6,100×. A profile was considered NK-1r immunoreactive (IR)

if it contained at least two gold particles. For each field, the following parameters were quantified: (*i*) the total number of NK-1r-IR and non-IR profiles of dendrites and cell bodies, (*ii*) the number of SP appositions and synapses on NK-1r-IR and non-IR dendrites and cell bodies, (*iii*) the perimeter of NK-1r-IR and non-IR profiles of dendrites and cell bodies, and (*iv*) the length of appositions of SP-IR boutons on NK-1r-IR and non-IR dendrites and cell bodies. To obtain the above parameters, the photographic negative plates from the electron microscope were placed on a light box, and the images were captured into an image analysis system (MCID M4, Imaging Research, St. Catharines, ON, Canada) by using a charge-coupled device camera. Data for each field were pooled per lamina and per Epon block, and the number of SP appositions and synapses per profile, as well as the percent of profile perimeter apposed by SP, was determined. Statistical analysis of the data between NK-1r-IR and NK-1r non-IR profiles per lamina was performed by using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

RESULTS

As reported (18), the highest densities of SP-IR fibers and varicosities were observed in lamina I and outer lamina II (lamina IIA), although immunostaining also was observed in inner lamina II (lamina IIB), especially its dorsal part, and in lamina III (Fig. 1*A*). In this study, the laminar distribution of the NK-1r immunoreactivity clearly matched the layer distribution of SP immunoreactivity (Fig. 1B) and corresponded mainly to dendrites and a few cell bodies. NK-1r-IR profiles occurred with the highest densities in laminae I and IIA, although considerable immunostaining was observed in lamina IIB and some in lamina III as well.

At the ultrastructural level, NK-1r immunoreactivity was observed in laminae I, IIA, IIB, and III (Fig. 2 *A–D*). The

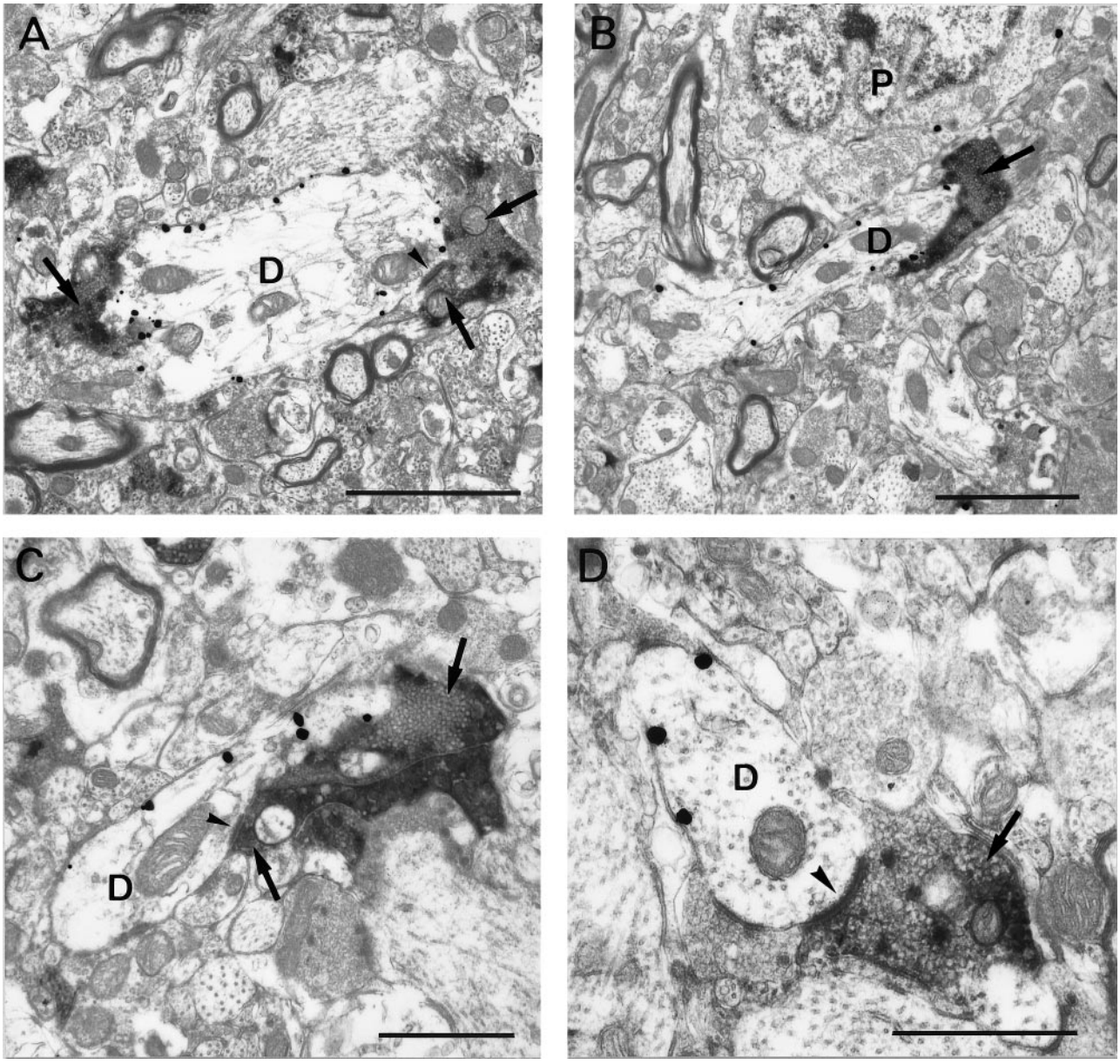

FIG. 2. Electron micrographs of the association between NK-1r-IR dendrites and SP containing boutons in laminae I-III of the rat spinal dorsal horn. *A*, *B*, *C*, and *D* demonstrate double-labeling for SP and NK-1r in lamina I, outer lamina II, the border between outer and inner lamina II, and lamina III, respectively. Note that several SP-IR boutons (arrows) are presynaptic to NK-1r-IR dendrites (D). Note also the presence of synaptic specialization's (arrowheads) between SP-IR boutons and NK-1r-IR dendrites. In *B*, note the presence of an NK-1r-IR cell body (P). [Bars = 2 μm (*A* and *B*), = 1 μm (*C* and *D*).]

immunoreactivity was mainly associated with the plasma membrane of dendrites and of a few cell bodies. No NK-1r-IR axons were detected. Axons containing immunoreactivity for SP established many appositions on NK-1r-IR profiles in all superficial laminae, and, often, synaptic contacts with the post-synaptic profile were observed.

Quantitative analysis revealed that NK-1r-IR profiles were a minority of the total population of dendritic/cell body profiles in all laminae. Values ranged from 13.3% of the total population in lamina I to 4.8% in lamina IIB. The number of SP appositions per dendritic/cell body profile was quantified and revealed that SP-IR boutons apposed a considerably higher number of NK-1r-IR profiles than those profiles not expressing the NK-1r in all three superficial laminae (Fig. 3*A*).

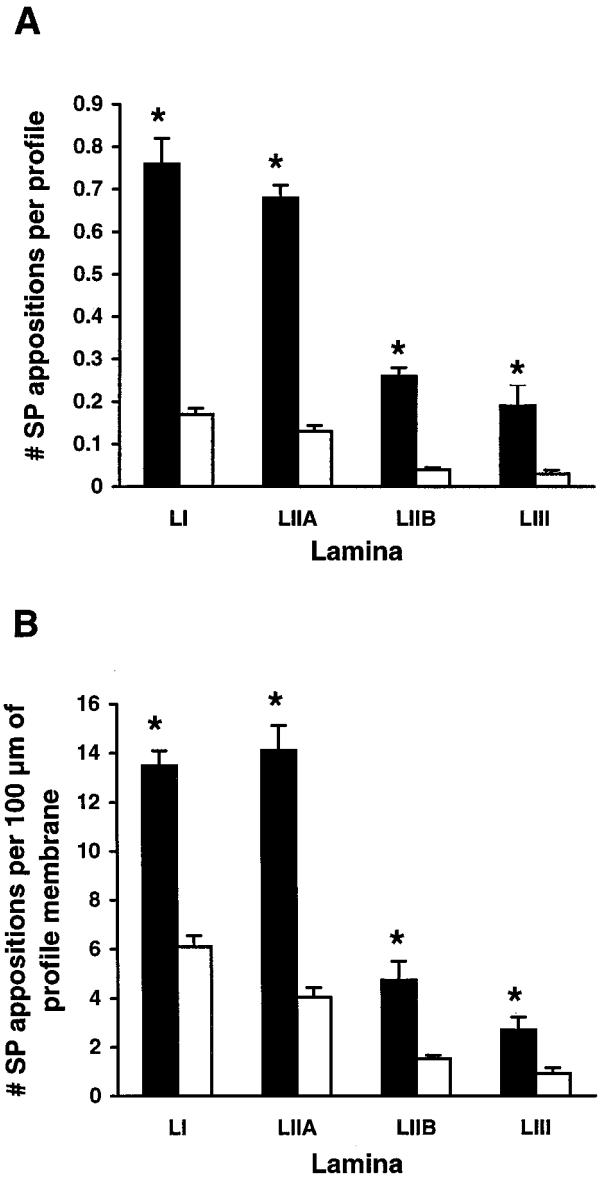

FIG. 3. Quantitative analysis of the association between SP-IR boutons and NK-1r-IR (■) or NK-1r non-IR (□) dendritic/cell body profiles in laminae I-III of the rat spinal dorsal horn. In *A*, it is shown that, in all laminae studied, the NK-1r-IR profiles possess a significantly higher number of SP appositions than those profiles not expressing the NK-1r. In *B*, it is shown that, in all laminae studied, the NK-1r-IR profiles possess a significantly higher number of SP appositions per unit length of profile membrane than those profiles not expressing the NK-1r. Values represent the mean ± SEM ($P < 0.05$). Asterisks indicate significant differences when comparing values for NK-1r-IR and non-NK-1r-IR profiles.

Although there were fewer SP appositions on NK-1r-IR profiles in laminae IIB and III, the values were always higher than for those dendritic/cell body profiles not expressing the NK-1r. Quantification of the number of SP appositions per 100 μm of dendritic/cell body profile membrane showed that a considerably higher number of SP-IR boutons apposed the membrane of NK-1r-IR profiles than of non-NK-1r-IR profiles in all three superficial laminae (Fig. 3*B*). The percent of the dendritic/cell body profile membrane apposed by SP was higher for those profiles expressing the NK-1r (12.4% for lamina I) than for those profiles not expressing the NK-1r (4.1% in lamina I) in all superficial laminae and paralleled the trend observed for the number of SP appositions per dendritic/cell body profile. The number of SP appositions making a synaptic contact on a postsynaptic target was quantified, and it was observed that an average of 30% of the SP appositions possessed a synapse in laminae I and II.

DISCUSSION

This study revealed a close correlation between the laminar distribution of SP and its receptor in the superficial laminae of the dorsal horn and provided important information on the peptide-receptor mismatch problem. In fact, our results indicate that SP preferentially innervates dendrites and cell bodies that express the NK-1r in all superficial laminae of the dorsal horn. The light microscopic analysis of the distributions of SP and the NK-1r in serial sections showed that the anatomical distribution of the peptide, SP, and the NK-1 receptor correlates well. In fact, both SP and NK-1r immunoreactivities were higher in laminae I and IIA and lower in laminae IIB and III. This correlation was confirmed at the ultrastructural level, where we found that profiles expressing the NK-1r were innervated preferentially by SP-IR boutons. Therefore, on the basis of these observations, a good peptide-receptor match in the spinal cord should be considered as the norm. We further suggest that the previously reported mismatch between the distributions of SP and the NK-1r when using certain antisera is caused by the failure in detecting some forms or antigenic presentations of the NK-1r that are not well understood at present, such as post-translational modifications of the receptor molecule (e.g., phosphorylation). However, the differences between the immunostaining patterns seen here and those shown in previous publications are not caused by the recognition of a short, carboxyl terminal truncated version of the NK-1 receptor (19–22) because the antiserum we used was generated against a peptide sequence that is absent from the short isoform.

It is important to point out that our electron microscopic analysis revealed many NK-1r-IR profiles apposed by SP-IR boutons in all superficial laminae, even in laminae IIB, a lamina in which other studies detected a low number of NK-1r-IR structures (8, 23, 24). Although we clearly detected more immunolabeling for the NK-1r in lamina II than in previous reports (8, 23), particularly in inner lamina II, such labeling corresponded almost exclusively to dendritic processes. Therefore, our observations concur with those of others (8, 23, 24) regarding a scarcity of NK-1r containing neuronal cell bodies in inner lamina II but differ substantially in the amount of immunolabeling in the same lamina.

As in previous studies (12, 25, 26), we observed that approximately one-third of the appositions between SP-IR bouton and NK-1r-IR profiles displayed a visible synaptic specialization. Although this value is apparently low, we should keep in mind that the synapses were observed in isolated sections. A much higher percentage, likely close to 100%, would have been observed if each individual SP-IR bouton had been examined in serial sections. In fact, a study by Beaudet and Sotelo (27) investigated the synaptic connections established by boutons onto surrounding profiles in the cerebellar

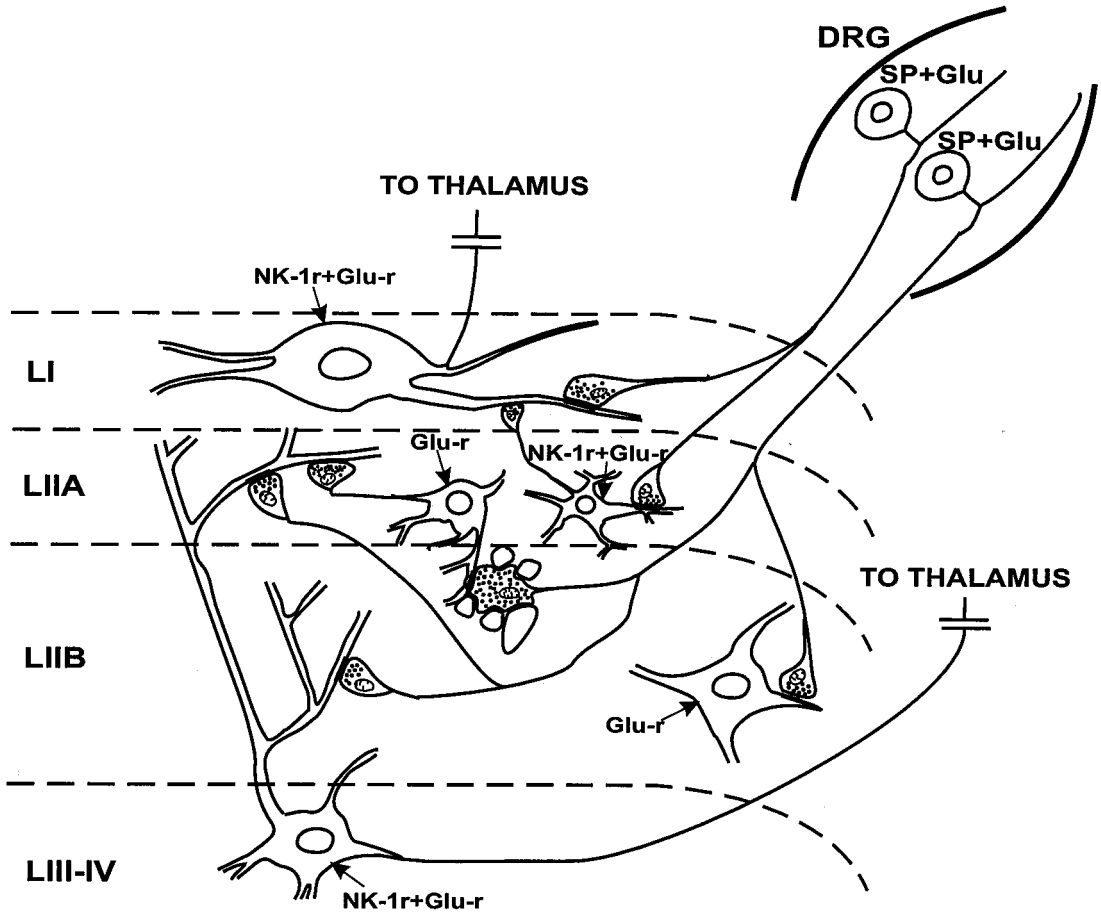

FIG. 4. Diagrammatic representation of the proposed synaptic circuitry involving SP-containing primary sensory fibers and dorsal horn neurons expressing the NK-1r. Because it is known that primary sensory fibers that contain SP also contain glutamate (SP + Glu), we propose that they have two types of synaptic targets in the superficial laminae of the dorsal horn. One type corresponds to neurons projecting to higher levels with cell bodies located in laminae I-IIA and laminae III-IV (see, for example, ref. 35) and that colocalize NK-1r and glutamate receptors (NK-1r + Glu-r). The other target corresponds to interneurons that express only glutamate receptors (Glu-r) located in lamina II. SP would activate the former and not the latter group of neurons, and glutamate would activate both types of neurons.

cortex by using serial sections. They concluded that, when 35% of the boutons displayed a synaptic specialization in isolated sections, this figure corresponded to 100% of boutons displaying a synaptic specialization after serial section analysis. Our study cannot be compared entirely with the one of Beaudet and Sotelo because a different area of the CNS was used and we investigated synapses with only NK-1r-IR profiles, and not all surrounding profiles. Because we demonstrated that ≈30% of the SP-IR boutons possessed a synapse in our paradigm, we can conclude that most, if not all, of the SP-IR boutons should be presynaptic to NK-1r-IR dendrites and cell bodies. In consequence, it is not unreasonable to state that the words apposition and synapse might have the same meaning because each apposition very likely corresponds to a synapse.

SP-IR boutons were also presynaptic to some profiles not expressing the NK-1r. This might represent the inability of the preembedding immunogold technique of revealing all postsynaptic structures that might express the NK-1r. Although this technique is reliable for detecting immunoreactivity (28), we cannot rule out that low levels of the receptor were not detected. However, an absence of detection in our study is unlikely because the NK-1r immunoreactivity was generally strong. We also should keep in mind that SP in the dorsal horn is colocalized with glutamate (29). The SP-IR boutons apposing non-NK-1r-IR profiles release not only the peptide but glutamate as well. It is possible that such postsynaptic targets express preferentially glutamate receptors. Fig. 4 represents a diagrammatic representation of this hypothesis. The confirmation of such a hypothesis would require the demonstration of glutamate receptors on the dendritic profiles apposed by SP that do not express the NK-1r.

The close association observed between dendritic/cell body profiles expressing the NK-1r and boutons containing SP, as well as the observation that SP is presynaptic to a higher number of NK-1r-IR profiles than of non-NK-1r-IR profiles, provides strong evidence that SP preferentially innervates postsynaptic targets expressing the NK-1r. These results concur and extend those from a confocal microscopic study by Naim *et al.* (11) that demonstrated a higher density of SP-IR varicosities onto neurons expressing the NK-1r than onto cholinergic neurons not expressing the NK-1r. Their findings, along with our previous observations demonstrating that nociceptive-specific neurons receive a high density of SP appositions as compared with non-nociceptive neurons (12, 25), favor the concept that the NK-1r expressing profiles apposed by SP-IR boutons are from nociceptive neurons. Conversely, those profiles not expressing the NK-1r and receiving few appositions with SP-IR boutons would very likely represent dendrites from non-nociceptive neurons. These results favor the hypothesis that there is a specific and highly organized anatomical and functional arrangement between varicosities from SP-containing fibers and targets expressing the NK-1r. A corollary of the findings would be that SP neurotransmission occurs across a very short distance rather than in a "diffuse" manner, as suggested by some groups (8, 30, 31). If this proves to be correct, peptide transmitters/modulators should behave

in a manner similar to that of classical neurotransmitters such as glutamate and glycine (32–34).

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