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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 central nervous system. In the present study we used an antiserum raised against a synthetic peptide corresponding to the carboxyl-terminal tail of the substance P (SP) 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-labeled SPR-binding sites in the central nervous system. 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 electron microscopic labeling 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 targeted receptor; neurotransmitter and receptor are separated only by the synaptic cleft and density between pre- and postsynaptic elements (1–4). Examples include glycinergic (3, 4) and some glutamatergic synapses (1, 2) in the central nervous system (CNS) and cholinergic synapses in sympathetic ganglia (5) and at the adult neuromuscular junction (6). On the other hand, some γ-aminobutyric acid (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 "nonsynaptic" fashion. The fact that postsynaptic densities are absent at some CNS monoaminergic synapses (7) suggests that norepinephrine and serotonin have a similar action.

Peptide neurotransmitters, which often colocalize with more classical neurotransmitters (8), may also act in a diffuse, nonsynaptic manner. Thus, for example: (*i*) there are significant mismatches between the distribution of peptides and their respective binding sites (9–11); (*ii*) peptide neurotransmitters can diffuse away from their site of release (12, 13) and can even be recovered in spinal cord cerebrospinal fluid (14); (*iii*) binding sites for μ (15) and δ (16) opioid peptides and for neurotensin (17) rarely overlap synaptic densities; (*iv*) dense core vesicles that contain neuropeptides are usually located away from the synaptic density, which is the presumed site of release of classical neurotransmitters (18, 19); and (*v*) the locus of exocytosis of dense core vesicles can, in fact, be distant from the synaptic junction (density) (20, 21). In the present report we use an antiserum directed against the substance P (SP) receptor (SPR), which corresponds to the NK-1 subtype of tachykinin receptors (22), and demonstrate that there is indeed significant mismatch at the synaptic level between peptide and peptide receptor. Furthermore, we demonstrate that the SPR 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 (240–260 g) that were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused through the ascending aorta with 100 ml of 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) followed by a 0.1 M sodium 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 and Minson (23). After the perfusion the brain and spinal cord were removed and postfixed in the same solution for 2–4 hr.

The autoradiograms of bound ^{125}I-labeled SP (^{125}I-SP) were generated as previously described (22). For immunocytochemistry, we used an anti-SPR antibody that was raised against a 15-amino acid peptide sequence [SPR-(393–407)] at the carboxyl terminus of the rat SPR (24). The immunogen consisted of synthetic peptide conjugated to bovine thyroglobulin by using glutaraldehyde. The antiserum used in this study (no. 11884-5) recognized a protein band of 80–90 kDa 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 SPR-(393–407). 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.* (25) was used (see below). Some light microscopic immuno-

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Abbreviations: CNS, central nervous system; GABA, γ-aminobutyric acid; SP, substance P; SPR, SP receptor.

cytochemical studies were performed on 15-μm cryostat sections of the brain (Fig. 1 *A* and *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, 70-μm-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 (23), washed in PBS, and then blocked in 10% normal goat serum for 1 hr. Next the sections were incubated in the SPR antiserum for 72 hr at room temperature. After extensive washing, the sections were incubated in a biotinylated secondary antibody for 2–4 hr and the avidin–biotin-peroxidase complex (Vectastain, Burlingame, CA) for 1 hr. To identify the immunoreaction product, the horseradish peroxidase 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 SPR-(393–407). These sections contained no immunoreaction product (data not shown).

To localize the SPR immunoreactivity at the electron microscope level, selected immunoreacted Vibratome sections through the cerebral cortex, striatum, and spinal cord were osmicated (0.5% OsO_4) for 1 hr, stained *en bloc* 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 SP- or GABA-immunoreactive terminals to study their synaptic relationship with SPR-immunoreactive profiles. Briefly, after washing in Tris-buffered saline, pH 7.6, containing 0.1% Triton X-100, the sections were incubated overnight in either a rabbit anti-SP (1:2000) or a rabbit anti-GABA antiserum (1:4000), both from Incstar (Stillwater, MN). After washing, the sections were incubated for 1 hr in a 15-nm colloidal gold-labeled goat antirabbit IgG (Amersham). 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 Electron Microscopy Laboratory and measured the perimeters of labeled and unlabeled membrane and the percentage of labeled membrane apposed by synaptic profiles.

RESULTS

General Distribution of the SPR Immunoreactivity. The distribution of SPR immunoreactivity was very similar to that reported in previous ^{125}I-SP-binding studies (refs. 22 and 26; Fig. 1 *A* and *B*). A detailed description will be published elsewhere. The receptor is located in some regions that have high concentrations of SP—e.g., superficial laminae of the dorsal horn of the spinal cord (Fig. 2*C*) and the locus ceruleus—and in some areas that have minimal peptide—e.g., cerebral cortex (Fig. 1 *B–F*). Consistent with the radioligand-binding studies, we detected no SPR 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 SPR Immunoreactivity. Unappreciated in light microscopy 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 (Fig. 1 *C–F*) and spinal cord (Fig. 2*C*); in other cases—e.g., the striatum (Fig. 1*B*) 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 labeling was also apparent. Although a similar pattern of labeling has been described in the striatum when a different antiserum was used (27), the authors of that study did not comment on the Golgi-like images that are produced.

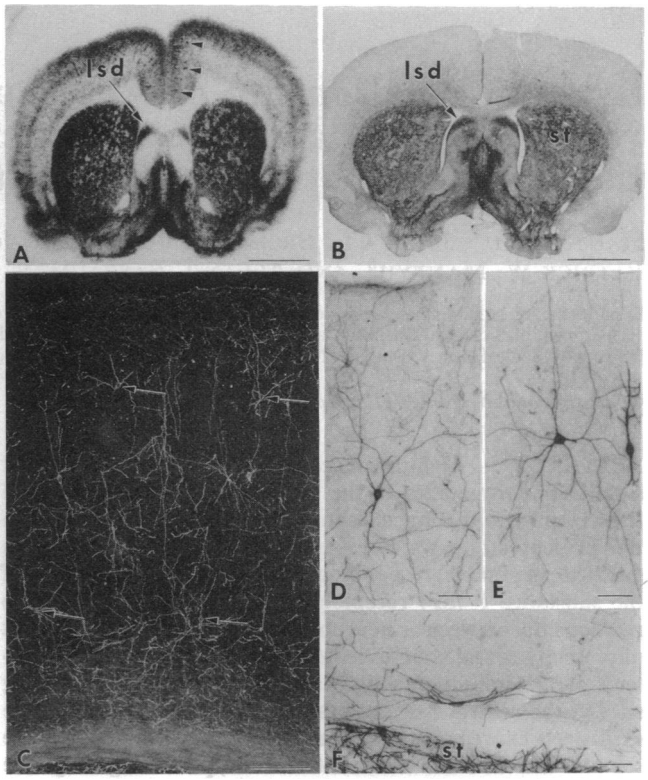

FIG. 1. *A* and *B* illustrate the comparable patterns of SPR labeling in coronal sections of the rat cortex determined by autogradiographic localization of ^{125}I-SP-binding sites (*A*) and immunocytochemistry using the SPR antibody (*B*). This is particularly clear for the striatum (st) and for the 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 labeled 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*.)

Fig. 1 *A* and *B* compare the pattern of SP binding and SPR immunoreactivity in the cerebral cortex and the striatum. Although areas of concentrated binding in the cortex (arrowheads in Fig. 1*A*) 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

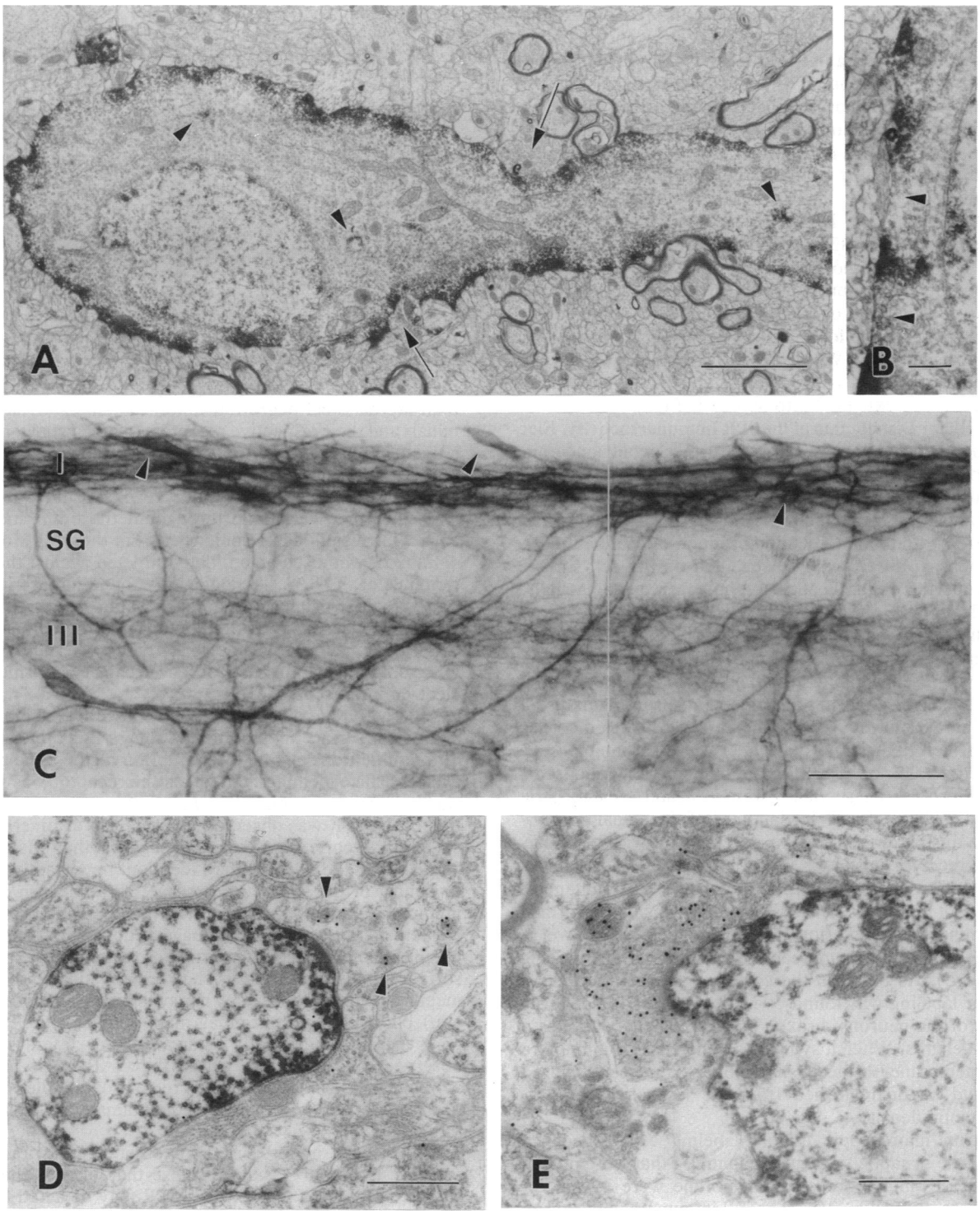

FIG. 2. The electron micrograph in *A* illustrates a cortical SPR-immunoreactive neuron. Although the SPR immunoreactivity is concentrated on the neuronal surface, a few cytoplasmic organelles are also labeled (arrowheads). Despite the dense labeling, only two synaptic boutons contacted the cell body in this section (arrows). *B* illustrates the alternating patches of labeled and unlabeled (arrowheads) surface membrane. The sagittal section of the lumbar spinal cord (*C*) illustrates a densely labeled neuron in lamina III. The dendrites of this neuron extend through the substantia gelatinosa (SG) to lamina I, which contains numerous labeled cell bodies (arrowheads) and dendrites. The latter arborize in lamina I. The SG itself contains few labeled cell bodies. Electron micrographs *D* and *F*, taken from lamina I of the dorsal horn, illustrate that both SP-immunoreactive terminals (*D*), identified by the colloidal gold labeling 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*.)

relatively small number of neurons (Fig. 1*C*). 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 (28), which have dorsoventrally arborizing dendritic trees (Fig. 1 *D* and *E*) and are often GABA-immunoreactive (29). 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 labeling in the dorsal horn of the spinal cord, where the organization of SP-containing terminals and SP-responsive neurons has been well characterized (30, 31), 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 (32). 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 SPR Immunoreactivity. Electron microscopic analysis provided further detail concerning the surface labeling of neurons. Fig. 2A illustrates an SPR-immunoreactive cortical neuron. Consistent with a report on the striatum (27), 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 labeling 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 labeled 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 (Fig. 2 D and 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 various lengths. When a synapse contacted SPR-immunoreactive membrane, the dense diaminobenzidine 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 (18, 19) and with the report that dense core vesicles exocytose at sites distant from the active zone (20, 21). Of 346 SP-immunoreactive terminals counted, 114 (33.3%) contacted an SPR-immunoreactive-laden profile, usually a dendrite. Finally, consistent with many other studies (33), 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 differences between the cellular and subcellular distributions of classical transmitter receptors and the SPR 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 SPR is indeed neuronal; in the normal rat it is minimally if at all associated with glial or other nonneuronal elements (34). 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 (3). 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.* (35), who used an antibody directed against the amino terminus of the SPR. Although they found intense immunoreactivity in the superficial dorsal horn, no cell body labeling was noted. They also reported axon and terminal, as well as dendritic, labeling. It will be important to determine whether the axonal labeling 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 percentage of surface membrane in which the receptor was inserted was uniformly 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 nonsynaptic 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 nonsynaptic regions of granule cells, was *never* contacted by the presumed glutamatergic mossy fiber terminals (36).

As has been hypothesized for other peptide transmitter systems (15), our results suggest that almost the entire surface of neurons that express SPR may be acted upon by SP, presuming that SP can diffuse a considerable distance from its site of release. In fact, Duggan and colleagues (12) 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 (13) or by calcitonin-gene-related peptide, which occurs with SP in primary afferent terminals (19) and which also retards SP degradation (37). 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 (38). By contrast, although neurons in laminae III and IV of the spinal cord typically do not respond to noxious stimulation (32), some express surface receptors that, if functional, can respond to SP (Fig. 2*C*), 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 nonnociceptive 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 (39) 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 SPR are functional targets of released transmitter. It is of interest, in this regard, to address the significance of the unlabeled 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 (40, 41), 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. This question can be addressed by 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 they reinforce the idea that cooccurring neurotransmitters in a synaptic terminal can target different postsynaptic elements.

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