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The Correlation Between the Distribution of the NK_1 Receptor and the Actions of Tachykinin Agonists in the Dorsal Horn of the Rat Indicates That Substance P Does Not Have a Functional Role on Substantia Gelatinosa (Lamina II) Neurons

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The presence of substance P in primary afferents that terminate in the outer laminae of the spinal cord has led to considerable interest in the function of this neuropeptide in nociception. We have examined the actions of tachykinin agonists on the membrane potential of neurons in lamina II of a neonatal spinal cord slice preparation *in vitro*. Only 10.5% ($n = 75$) of these neurons showed any response to the application of a selective NK_1 receptor agonist while 48.3% ($n = 60$) of neurons in deeper dorsal horn laminae responded to this agonist. Lamina II neurons were equally insensitive to selective NK_2 and NK_3 agonists. Synaptic potentials evoked in lamina II neurons by peripheral nerve stimulation were similarly not altered by the NK_1 agonist. Immunocytochemical studies using an antibody raised against the C-terminal of the NK_1 receptor revealed that very few lamina II neurons express NK_1 receptors, and this offers an explanation for our findings.

[Key words: tachykinins, neurokinins, substance P, substantia gelatinosa, lamina II, spinal cord, dorsal horn]

The tachykinins substance P and neurokinin A (NKA) are both localized within small-diameter primary afferents (Hökfelt et al., 1975a,b; Barber et al., 1979; Dalsgaard et al., 1985; Ogawa et al., 1985) that terminate predominantly in the substantia gelatinosa (SG) of the spinal cord (Barber et al., 1979) and trigeminal nucleus caudalis (Salt et al., 1983; Priestley and Cuello, 1989). Noxious intensities of peripheral stimuli cause the release of substance P in the SG (Duggan and Hendry, 1986). In addition to its distribution in primary afferents, substance P has also been localized within small numbers of spinal projection neurons (Leah et al., 1988; Conti et al., 1990) and in descending axons from the region of the raphe magnus (Johansson et al., 1981). Additionally, colchicine treatment of the spinal cord reveals the presence of many substance P–positive cells in lamina II (Conti et al., 1990).

Tachykinin (neurokinin) receptor subtypes have now been cloned and sequenced (e.g., Hershey and Krause, 1990; see Gerard et al., 1993, for review) and a range of selective agonists for these receptors are available (Dion et al., 1987; Regoli et al., 1988). With the development of *in vitro* spinal cord slice preparations from which long-term stable intracellular recordings can be made throughout the dorsal horn, it has become possible to study synaptic transmission in the SG in more detail (e.g., Yoshimura and Jessell, 1989a,b). The present study was initiated to investigate the direct actions of selective tachykinin receptor agonists on the membrane potentials and primary afferent evoked synaptic responses of SG neurons. In view of the negative results obtained in preliminary experiments the effects of these tachykinin agonists on deeper dorsal horn neurons was also investigated. Similar negative findings for the action of Substance P on SG neurons in spinal cord slices form adult rats have recently been reported (Yoshimura et al., 1993). The recent production of an antibody to the C-terminal sequence of the rat NK_1 receptor (Vigna et al., 1994) has permitted mapping of its distribution (Brown et al., 1993; Liu et al., 1993, 1994; Vigna et al., 1994), and we have used this antibody to locate the NK_1 receptor in neonatal rats of the same age as those used in electrophysiological studies. This supports the conclusion from our electrophysiological data that neurons in deeper laminae but not SG have functional NK_1 receptors and this has far reaching implications for the understanding of the function of the SG.

A preliminary report has been made of some of the electrophysiological data reported here (Bleazard and Morris, 1993).

Materials and Methods

Electrophysiology. After ether anesthesia, juvenile (12–20 d old) rats were decapitated and their vertebral columns dissected. All the dissection stages were carried out in a bath perfused with at least 5 ml/min with an artificial CSF (ACSF) that had the following composition (mM): NaCl, 120; KCl, 2.1; $KHPO_4$, 1.0; $MgSO_4$, 1.3; $NaHCO_3$, 25; $CaCl_2$, 2.4; glucose, 10; phenol red, 5 mg/liter; gassed with 95% O_2, 5% CO_2, at 4°C. The lumbar cord with the attached, right side, L3–L5 dorsal roots, dorsal root ganglia, femoral and sciatic nerves were dissected. The cord was then sliced using a modified Vibroslice (Camden Instruments) into transverse slices approximately 350 μm thick. Cuts were positioned just caudal and rostral to the dorsal roots to yield slices with some intact afferent input from peripheral nerves. The Vibroslice modification consisted simply of altering the blade holder to allow cuts to be made vertically instead of horizontally. Slices were arranged in a multicompartment tissue bath that permitted separate perfusion of the spinal cord slice and dorsal root ganglion, and isolated stimulation of the peripheral nerve. Peripheral nerve stimulation was via two pairs of platinum electrodes. After placing these in contact with the nerve, a warm (45°C) liquid paraffin–petroleum jelly mixture, with a setting temperature of 30°C, was applied to insulate between electrodes and stop

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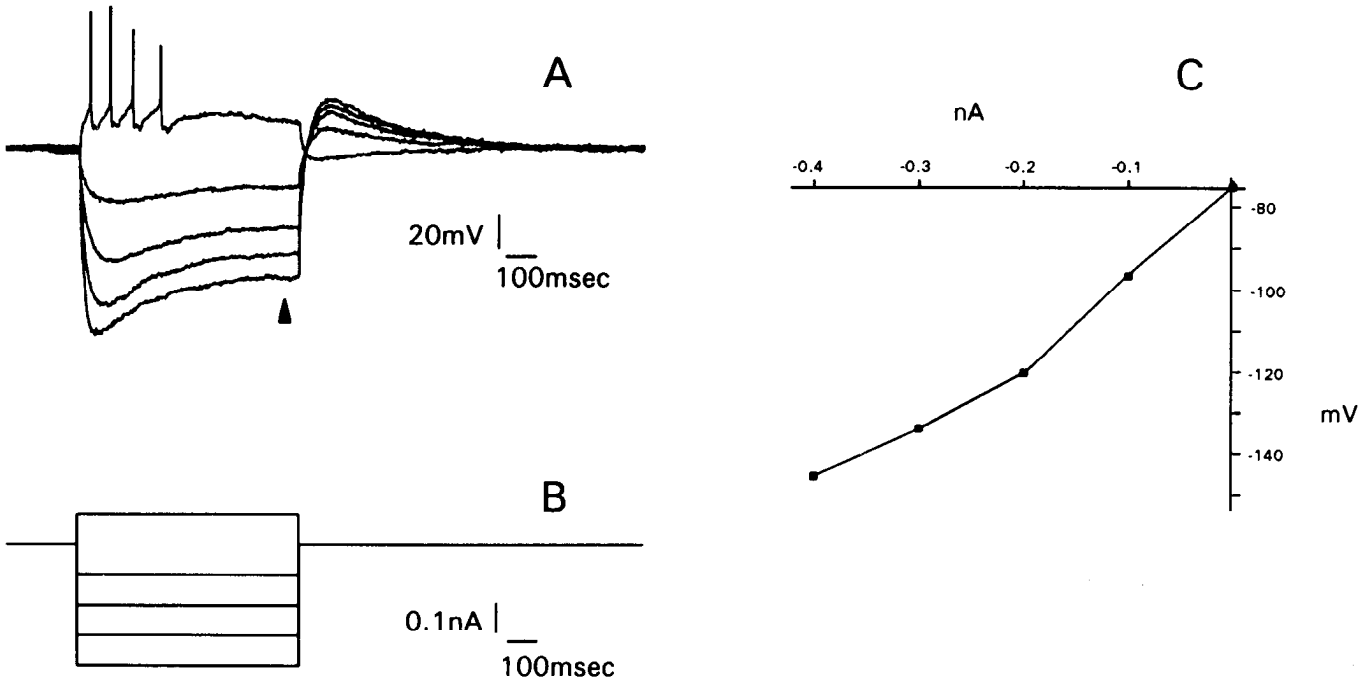

Figure 1. Responses of a typical SG neuron (membrane potential $V_m = -75$ mV) to current injection. *A*, Voltage responses to a series of current pulses as shown in *B*. The neuron shows a delayed rectification to prolonged hyperpolarization. The relationship between current and voltage for the hyperpolarizing steps at the point indicated by the *arrowhead* is plotted in *C*. Note the cell shows an overall inward rectification. On cessation of the hyperpolarizing current pulse the cell shows a rebound depolarization.

dehydration of the nerve. The slice and dorsal root ganglia were perfused with ACSF of the same composition as that used for dissection but at 28°C ± 1°C. In some experiments the effects of raising the perfusion solution temperature to 35°C was studied. The Mg^{2+} ion concentration was also lowered to a nominal zero in some experiments by omission of $MgSO_4$ from the ACSF. Intracellular recordings were made from neurons throughout the dorsal horn including the SG and lamina X. As the slices were relatively thin and were transilluminated, electrodes could be directly inserted under visual control into specific areas of the dorsal horn. The SG contains relatively few myelinated fibers and appeared as a translucent band that permits precise electrode targeting on this region. Electrodes were filled with 3 M potassium acetate or 2% Lucifer yellow dissolved in 1 M lithium chloride and had resistances of over 140 MΩ. Sample cells were intracellularly injected with Lucifer yellow to confirm their locations. Drugs, at a range of concentrations were made up in ACSF, and applied to the spinal cord slice by switching between the perfusion solution and the drug solution reservoirs. The following drugs were used in these studies: [Sar^9, Met$(O_2)^{11}$]-substance P (SarMetSP), [[βAla^8]-neurokinin A (4-10)] (βA-NKA), [Nle^{10}]-neurokinin A (NleNKA), [Suc-[Asp^6, MePhe^8]-substance P(6-11)], (Senktide), [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAGOL), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), and 3-((*RS*)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP).

Immunocytochemistry. Neonatal rats (12–19 d) were deeply anesthetized with urethane (1000 mg/kg) and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Sections were cut on a freeze knife microtome (40–80 μm) and processed free-floating. The primary antibody used to map the NK_1 receptor was a gift from Dr. S. Vigna. It was raised in the rabbit to the C-terminal 15 amino acid fragment (amino acids 393–407) of the rat NK_1 receptor (Hershey and Krause, 1990). Sections were incubated in this antibody, which was diluted 1:30,000 in PBS containing Triton X-100 (0.5%) and bovine serum albumin (BSA) (2.5%), for 12 hr at room temperature. Subsequently the binding of the primary antibody was detected using biotinylated anti-rabbit antisera (1:1000) and streptavidin-conjugated horseradish peroxidase (1:1000) (both from Amersham). Finally the chromogen protocol of Shu et al. (1988) was used to reveal the distribution of bound peroxidase.

Results

Electrophysiology

Intracellular recordings were made for periods of 30 min to over 3 hr from 135 dorsal horn neurons. Only cells with stable resting membrane potentials more negative than −55 mV and that produced action potentials to depolarizing current pulses are included in the data base. Seventy-five of the cells were made in electrode penetrations targeted on the SG while the remaining 60 were recorded from tracks targeted on deeper laminae (III–VII and X).

Membrane characteristics of SG neurons

The mean resting membrane potential of the SG neurons was −75 mV ± 1.1 mV (mean ± SEM, $n = 75$). Of these cells, six were confirmed as being located in lamina II through the histological localization of dye injected into the neuron. Depolarizing and hyperpolarizing current pulses were injected into sample SG neurons ($n = 7$) to compare their membrane properties with the detailed description given for SG neurons in slices from adult rats by Yoshimura and Jessell (1989b). Time-dependent anomalous rectification was a feature of the response of most of these cells to constant current hyperpolarizing pulses (Fig. 1). They also frequently had marked rebound depolarization on cessation of current injection (Fig. 1). In a few cells this time-dependent rectification was not apparent (see Fig. 4) but a small rectifying current was evident when current–voltage analysis was performed. In this study we have concentrated on the responses of SG neurons to tachykinin agonists; however, the limited biophysical measurements made suggest that the neonatal SG neurons investigated have similar properties to those previously described in the adult (Yoshimura and Jessell, 1989b).

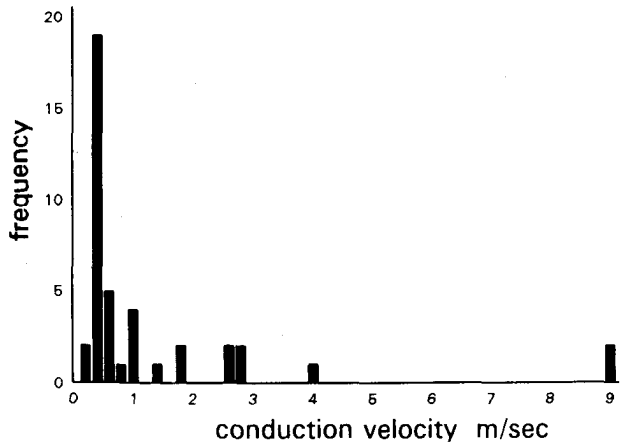

Figure 2. Graph of the distribution of conduction velocities for lamina II neuron ($n = 41$) synaptic responses. These were calculated from the latency to the beginning of an evoked response and the conduction distance between the stimulating electrode (proximal cathode) and recording electrode.

Primary afferent input to SG neurons

Peripheral nerve stimulation evoked responses in 41 SG neurons. Those that were found to be unresponsive could either have been deafferented by the slicing procedure or receive afferents from other peripheral nerves cut during the dissection. The activated neurons typically showed EPSPs with considerable variability in their duration and components. IPSPs were also observed and these frequently followed the excitatory responses and consequently reduced their duration. Conduction velocities for the primary afferent fibers evoking these responses were estimated (mean conduction velocity, 1.2 ± 0.3 m/sec, ±SEM; $n = 41$) (Fig. 2). Assuming that most of the early components of these responses are evoked monosynaptically these measurements would suggest that these responses are mediated by both Aδ- and C-fibers. Threshold measurements also suggest that both fiber types are involved. Responses evoked at long latency that gave calculated conduction velocities below 0.8 m/sec required high-threshold stimulation for their activation (mean, 44.8 ± 6.2 V, ±SEM; 0.5-msec-duration stimulus, $n = 18$). Stimuli of 0.05 msec duration were adequate to excite cells responding with latencies giving calculated conduction velocities above 1 m/sec. For those in which the threshold was tested with 0.5 msec duration stimuli, thresholds were typically below 20 V (mean, 17 ± 5.1 V, ±SEM; $n = 8$).

Effect of tachykinin agonists on SG neuron membrane properties

SarMetSP (100 nM to 1 μM) was applied for periods of 30 sec to 4 min to a total of 75 SG neurons. The tissue bath and perfusion system design resulted in a delay of 5 sec to the entry of drugs into the bath and a further 15 sec for the complete bath volume to change. Equilibrium conditions at the level of cells in the slice were estimated to have been achieved by 30 sec for drugs that are not subject to significant enzymic breakdown or uptake. For this reason stable analogs were used in preference to endogenous ligands. Of the 75 cells tested, only eight (10.5%) showed a clear and reversible change in response to SarMetSP application, and in most cases these were quite small. An additional three cells responded with an increase in firing during the application of SarMetSP but no recovery was obtained after wash out with drug free ACSF. Of the eight responsive cells four showed an increase in firing during drug application that in some cases was accompanied by a small depolarization (see Fig. 4). One cell showed an increase in EPSP frequency with a small depolarization. Two cells responded with an increase of IPSP frequency that became more readily observed on cell depolarization. The remaining cell showed a small increase in its evoked EPSP size but in view of the variability in the control EPSPs this may not have been significant. The vast majority ($n = 64$, 85.5%) of SG neurons showed no change in their membrane potential to application of the selective NK_1 receptor agonist (Fig. 3). Some experiments were carried out in nominally Mg^{2+}-free ACSF (see later). A similar percentage of cells responded to SarMetSP in the presence (10.4%, 7 of 67) or absence (12.5%, 1 of 8) of magnesium. Alterations in the temperature (raised to 35°C, $n = 3$) of the perfusion solution did not change the response properties of the cells significantly. Slices maintained at 35°C appear to have poor survival characteristics possibly due to the lower solubility of O_2 and higher metabolic rates at this temperature.

The actions of other tachykinin receptor agonists were tested on 21 cells that had been tested with SarMetSP (100 nM or 1 μM). The NK_2 agonists βA-NKA (1 μM) or NleNKA (1 μM), and/

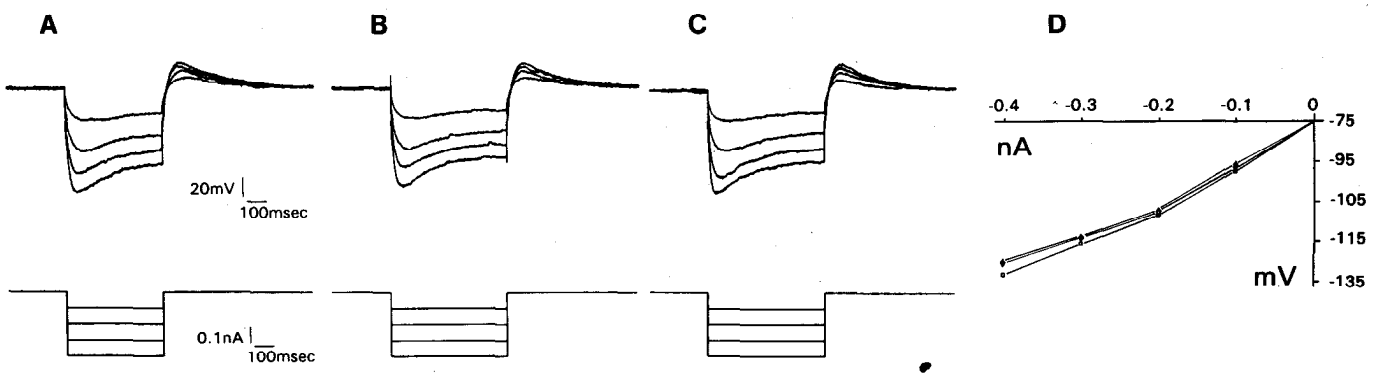

Figure 3. SarMetSP (1 μM) was applied to this cell for 2 min with no effect on the membrane potential. During a predrug control (*A*), toward the end of drug application (*B*) and in a postdrug, wash-out period (*C*) hyperpolarizing pulses were injected into the neuron. These produced almost identical current–voltage relationships (*D*). Measurements were made toward the end of the hyperpolarizing pulses as illustrated in Figure 1 ($V_m = -75$ mV).

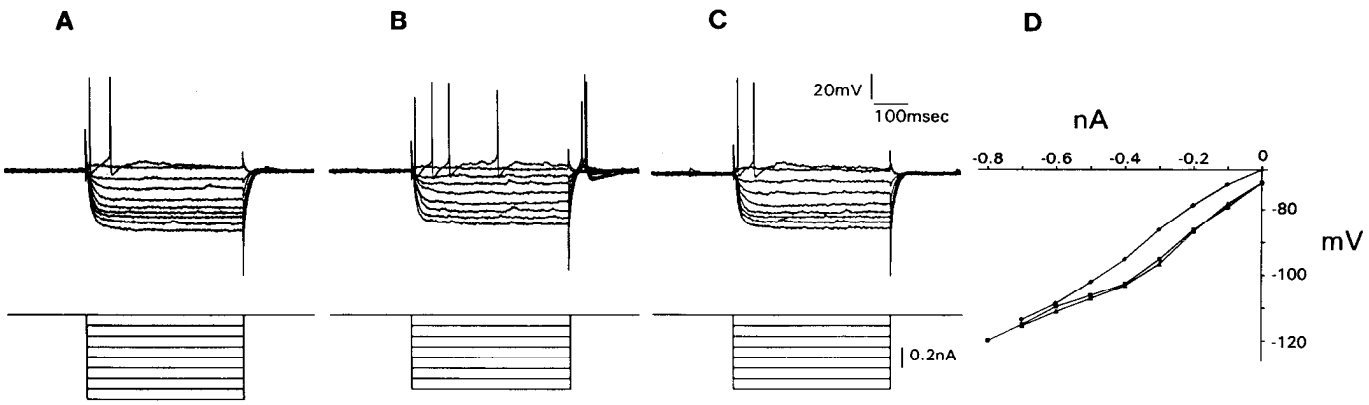

Figure 4. Lamina II cell showing weak action of SarMetSP. This cell was tested in a similar manner to the cell illustrated in Figure 3. *A* and *C* show pre- and postdrug current–voltage tests, and *B*, the response toward the end of drug application. SarMetSP produced a small overall depolarization and increased cell firing. This was seen most clearly when a 0.1 mA depolarizing pulse was applied to this cell (*upper trace in A–C*, not shown in current injection records). The current–voltage plots (*D*) show a clear effect of SarMetSP (*upper graph line*) at membrane potentials more than −110 mV (V_m = −72 mV).

or the NK_3 agonist Senktide (100 nM and 1 μM) were used. Of these 21 cells 16 of 21 (76%) were unresponsive to SarMetSP while 5 of 21 (24%) responded. Of the group that showed no response to SarMetSP only one cell showed any response to the other agonists (15 tested with NK_2 agonists and 8 with senktide). The one responsive cell showed a small increase in EPSP frequency during the application of senktide. The five cells that were responsive to SarMetSP were all tested with βA-NKA. Three showed similar responses to application of both agonists (two showing depolarization and an increase in firing or EPSPs, one increase in IPSP frequency) while two were unaffected by βA-NKA (both of these were excited by SarMetSP). One cell that was depolarized and increased its firing frequency in response to both SarMetSP and βA-NKA was similarly excited by senktide.

Effects of tachykinins on SG neuron synaptic responses mediated by excitatory amino acids

In view of results demonstrating that substance P acts on deeper dorsal horn neurons by altering their responses to NMDA (Dougherty and Willis, 1991; Rusin et al., 1992, 1993) we have looked for evidence for the interaction of tachykinin agonists with the excitatory amino acid mediated synaptic responses of SG neurons. In agreement with other reported results (e.g., Schnieder and Perl, 1988; Jeftinija, 1989; Yoshimura and Nishi, 1992, 1993) we have found that the EPSPs evoked by peripheral nerve stimulation in SG neurons can usually be blocked by excitatory amino acid antagonists. In the absence of magnesium most of the evoked EPSPs are prolonged and have components that can be blocked by selective antagonists acting at AMPA receptors such as CNQX (2 μM) and NBQX (2 μM) and at NMDA receptors such as CPP (1–10 μM) (Fig. 5*A*). In the presence of 1.3 mM Mg^{2+} the EPSPs evoked by single stimuli applied to the peripheral nerve were typically short and almost totally blocked by antagonist acting at AMPA receptors (Fig. 5*B,C*). The effects of SarMetSP on EPSPs were examined in the presence (n = 30) and absence (n = 7) of magnesium. No consistent change in the EPSPs amplitude, duration or shape was observed that could be interpreted as a selective facilitation of the NMDA component of the EPSP (Fig. 5*D–F*). In 33 (89%) cells the EPSP showed no change during the application of SarMetSP while a further three cells showed an overall reduction of the EPSP and one showed some facilitation.

Effects of DAGOL on SG neurons

In view of the preponderance of negative findings for the actions of these tachykinin agonists on SG cells we tested another peptide acting via an alternative G-protein linked receptor that is known to have actions on such neurons (Jeftinija, 1988). In 5 out of 7 neurons tested, the μ-opiate receptor agonist DAGOL (1–10μM) caused a membrane hyperpolarization. In three out of four of these neurons in which responses were also evoked by peripheral nerve stimulation the evoked EPSPs were reduced in amplitude or abolished.

Effects of tachykinin agonists on deeper-lamina neurons

SarMetSP was applied to a further 60 neurons in laminae deeper in the dorsal horn. Of these, 29 (48.3%) responded to SarMetSP (100 nM to 1 μM). The majority of these were excited (n = 25) by way of an increase in action potential and/or EPSP frequency, sometimes accompanied by an overall membrane depolarization (Fig. 6). In four neurons the response consisted purely of an increase in the frequency of IPSPs. In some slices, SarMetSP was found have no action on an SG neuron while readily exciting a deeper-lamina neuron.

Immunocytochemistry

NK_1 receptor staining was seen throughout the dendrites and cytoplasm of the somata of neurons in the dorsal horn and around the central canal (X) (Fig. 7*A,B*). In many neurons the staining appeared denser close to the plasma membrane. This pattern of staining suggests that the NK_1 receptor is distributed widely over the cell surface and is not restricted to subsynaptic sites (see also Liu et al., 1993, 1994). Due to this widespread staining it was possible to build a detailed picture of the organization of the neurons expressing this receptor. Many are located in lamina I (Fig. 7*E*) and have dendrites that run in a mediolateral plane through this lamina with some running a little deeper into the outer part of lamina II. The majority of lamina II cells did not show any staining with this antibody (Fig. 7*A–D*) and the stained dendrites in this lamina could usually be traced back to cells in lamina I or deeper in laminae III–

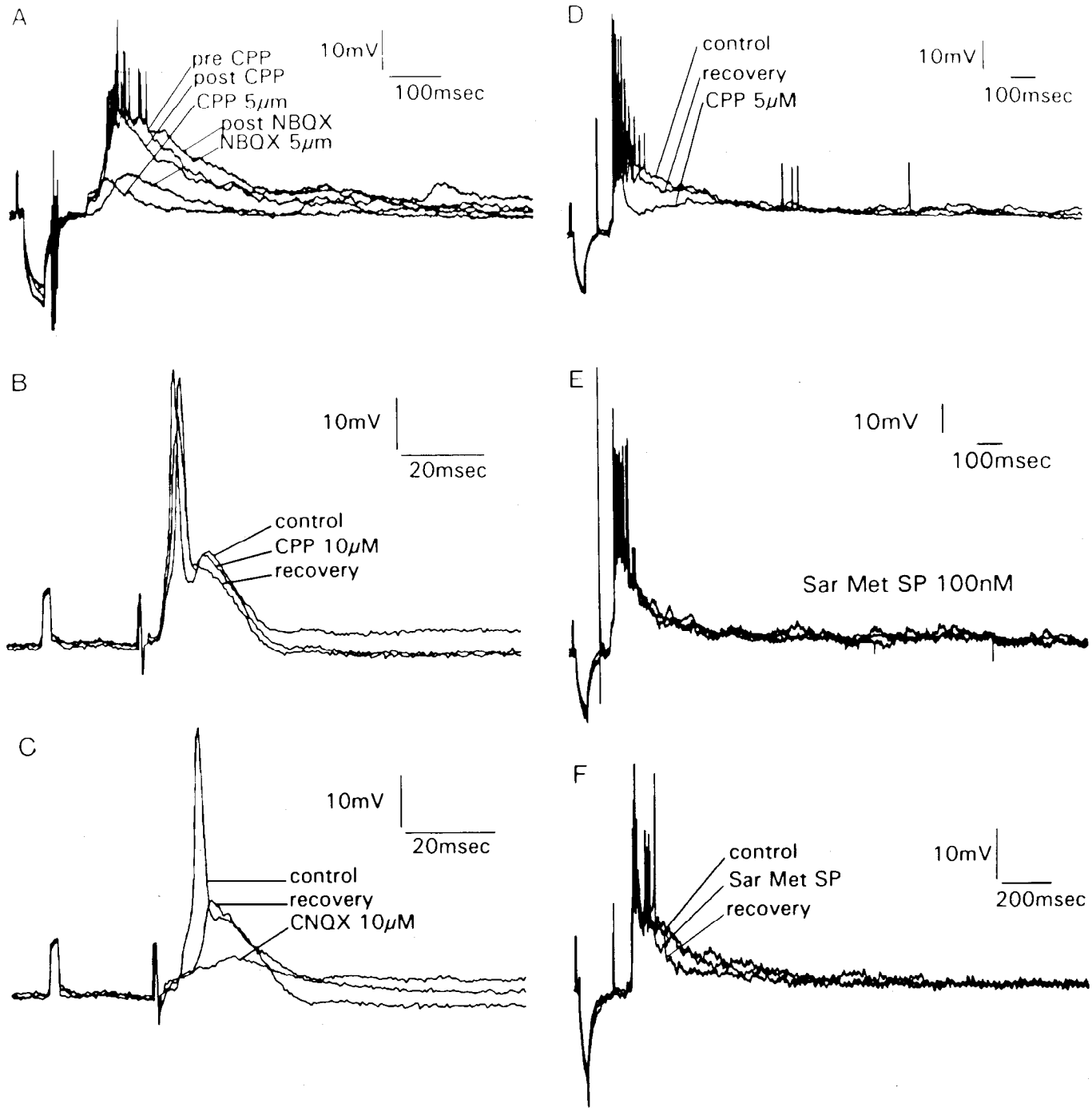

Figure 5. Averages (five responses each trace) of EPSPs evoked in lamina II neurons and effects of drugs. *A*, In Mg^{2+} free ACSF EPSPs evoked by peripheral nerve stimulation were prolonged. In the presence of NBQX a smaller depolarization with a slower onset remained. In the presence of CPP a fast depolarization persisted. This indicates that both AMPA and NMDA receptors mediate these responses. (In *A* and *D–F* the stimulus is preceded by a short hyperpolarizing pulse that was used to observe conductance changes. Also, a short 10 mV calibration pulse is present at the start of each trace). *B* and *C* (same cell), In the presence of Mg^{2+} the overall EPSP is typically much shorter and CPP (*B*) has little effect whereas CNQX (*C*) almost totally blocks the response. *D* and *E* (same cell), Response in the absence of Mg^{2+} shows a large component blocked by CPP (*D*). This cell was also tested with 100 nM SarMetSP but showed no alteration (pre- and postcontrols are superimposed on the SarMetSP response). *F*, This response was evoked in the presence of Mg^{2+} but is particularly prolonged. The potentials both prior to drug application (SarMetSP 100 nM) and during drug application were identical following drug wash out a small depression in the later components of the EPSP is apparent (V_m: *A*, −66 mV; *B* and *C*, −60 mV; *D* and *E*, −76 mV; *F*, −64 mV).

IV (Fig. 7*C,D*). Occasional large cells are seen in lamina II that stain for the NK_1 receptor; however, these have dendrites that enter lamina I and have the appearance of displaced lamina I cells. The lamina III–IV cells that stained with this antibody had a striking morphology with long dendrites radiating through lamina II (Fig. 7*D*). These dendrites gave rise to very few branches in lamina II but then branched extensively in lamina I (Fig. 7*D*). The dendrites of these cells appear to have few spines; however, the possibility that the receptor protein is not present in spines has to be kept in mind. In the lateral spinal nucleus,

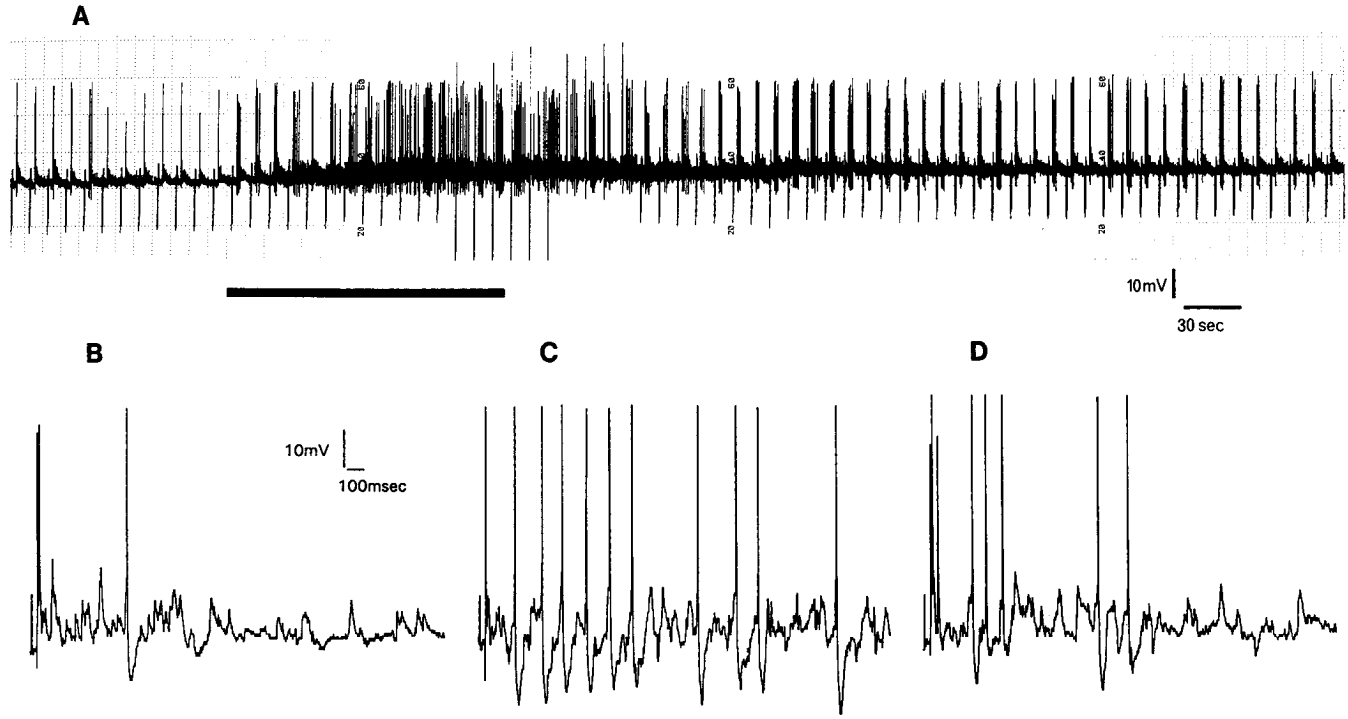

Figure 6. Response of a deeper-lamina neuron to SarMetSP application. *A*, Chart recorder trace of cell activity (the chart recorder frequency response maximum of 500 Hz causes the action potentials to appear reduced in amplitude). During the period indicated by the *solid bar* SarMetSP at 1 μM was applied. Shortly after the start of application the frequency of EPSPs increases and the cell starts to fire (peripheral nerve stimuli and hyperpolarizing conductance pulses are also being applied throughout this record, and toward the end of drug application a series of hyperpolarizing and depolarizing pulses were applied). Sample parts of the record are shown on a faster time base in *B–D*: *B*, prior to drug application; *C*, toward the end of drug application; *D*, after partial recovery during the drug washout. From *trace C* it can be seen that the action potential frequency has increased while the overall membrane potential is almost unchanged ($V_m = -65$ mV).

a distinct plexus of stained neurites and cells was present (Fig. 7*F*). Around the central canal another cluster of receptor expressing cells was located. Throughout the deeper laminae of the dorsal horn (IV–VII) occasional cells expressed NK_1 receptor. At the thoracic level large numbers of intermediolateral horn cells were found to stain for the NK_1 receptor.

Discussion

The electrophysiological data are potentially open to a number of different interpretations. Initially, in view of the distribution of tachykinin containing primary afferents, we were predisposed to think that the lack of action of tachykinin agonists on SG neurons was a consequence of either developmental factors or technical problems. In view of our studies and those of other groups, in which the NK_1 receptor protein has been mapped using immunocytochemical techniques we would now explain the lack of response to NK_1 agonists by the absence of NK_1 receptors on SG neurons. We will, however, consider the other possible interpretations.

It is possible that SG neurons in rats of the ages used in this study, have not yet developed functional NK_1 receptors. We cannot exclude this possibility but several lines of evidence make it improbable. Behavioral experiments show that by 10–11 d postnatal, rats show reflex responses to a full range of noxious stimuli including chemical, thermal, and mechanical (Fitzgerald and Gibson, 1984). Isolated spinal cord–nerve preparations from 1–4-d-old rats have a slow component to their ventral root potentials, that is evoked by intense primary afferent stimulation and can be blocked by the peptide NK_1 receptor antagonist spantide (Nussbaumer et al., 1989). Both these observations suggest that at the reflex level rats develop functional NK_1 receptors in the first week after birth. In some of the present experiments, tachykinin agonists were applied to both an unresponsive SG and responsive deeper-lamina neuron in the same slice again demonstrating the presence of functional NK_1 receptors. Tachykinin binding sites have also been demonstrated in the SG by 8–15 d postnatally (Charlton and Helke, 1986). Additionally, immunocytochemical studies show the presence

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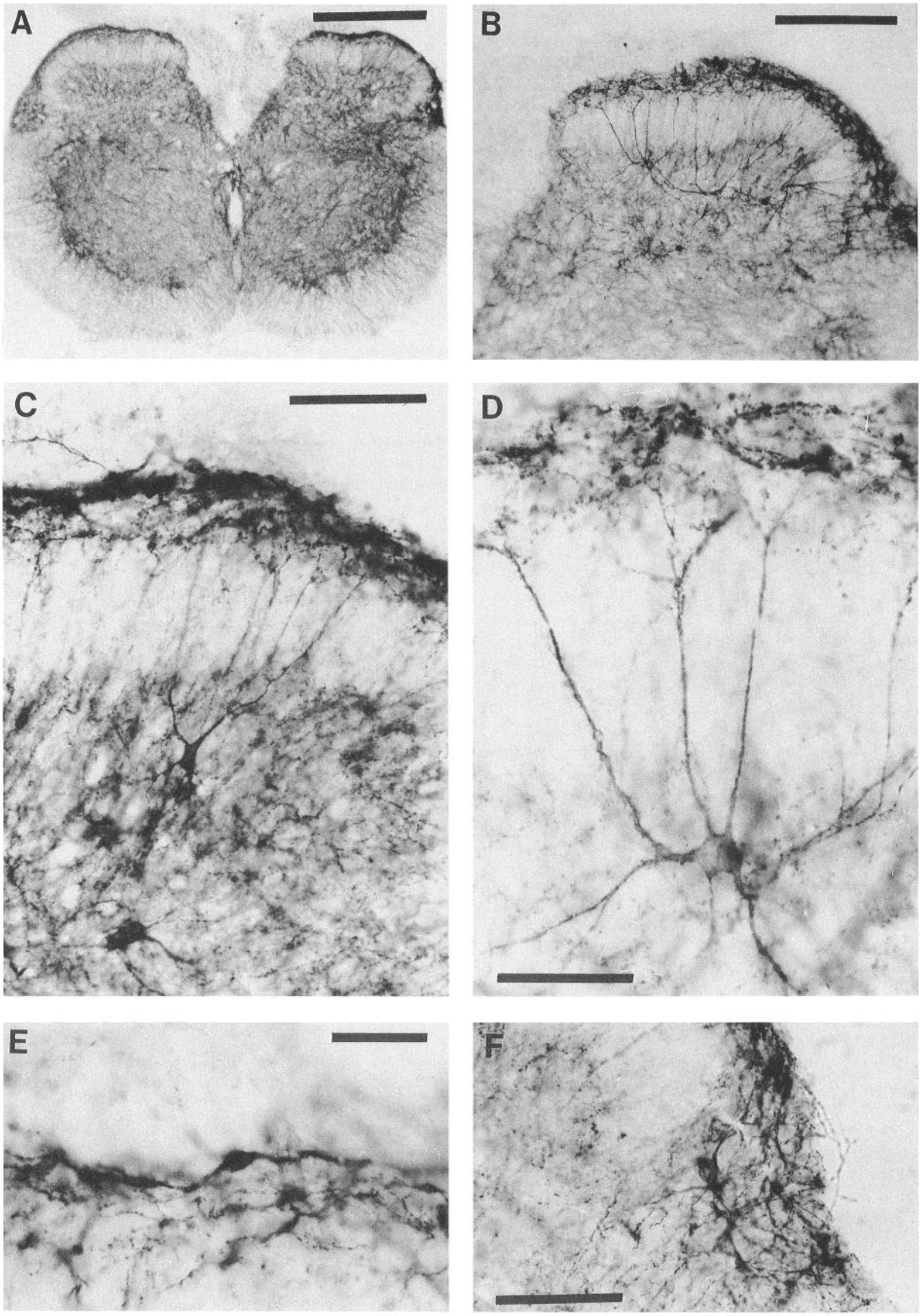
Figure 7. Immunocytochemical distribution of the NK_1 receptor. *A*, Low-power overview of staining in transverse lumbar spinal cord section. Staining is mainly confined to the gray matter, with a clear absence of staining in lamina II. *B*, At slightly higher magnification it can be seen that a dark band of staining is present in lamina I and more diffuse staining is present in lamina III and deeper. *C*, Intensely stained neurons are present in lamina III that give rise to dendrites that traverse lamina II. Lamina I shows a complex intensely stained neuropil. *D*, This lamina III neuron illustrates the typical features of this group of NK_1 receptor–bearing neurons. The soma gives rise to several dendrites, some of which distribute to deeper laminae. A number of dendrites radiate dorsally and cross lamina II characteristically without giving rise to extensive collateral branches. As they approach lamina I they branch more extensively. *E*, Many lamina I neurons are intensely stained and where their structure can be discriminated they appear bipolar with dendrites running medially and laterally. *F*, The lateral spinal nucleus is an area of intense NK_1 receptor staining in both cells and processes. Scale bars: *A*, 500 μm; *B*, 250 μm; *C* and *F*, 100 μm; *D* and *E*, 50 μm.


A
B
C
D
E
F

of substance P (Fitzgerald and Gibson, 1984) in the SG by a few days postnatally. Hence all available data would point to the presence of a functional tachykinin (presumably substance P) containing input to the dorsal horn in rats of this age.

With regard to the possibility that the receptors are present on SG neurons but are no longer functional due to the trauma of slice preparation, the principle concern is that anoxia may result in deterioration of receptor coupling to second messenger systems. Clearly by standard criteria of membrane potential, synaptic activation and current–voltage responses the SG cells studied respond in a manner that would suggest that no overt changes in their ionotropic responses are present. To examine the operation of functional second messenger mediated mechanisms, we tested the responses to DAGOL and neurons responded as would be predicted from previous studies reported in the literature (Jeftinija, 1988). Finally, the responses of deeper-lamina neurons to NK_1 agonists were as expected from the many precedent studies (e.g., Henry 1976) and particularly by Randic and coworkers (e.g., Murase and Randic, 1984). Due to the larger diffusion distance for oxygen during the dissection procedures, neurons toward the core of the spinal cord might be expected to be more susceptible to anoxic damage. In toluidine blue-stained semithin sections from hemisected spinal cord preparations the first signs of anoxic damage were always observed in deeper-lamina neurons (Morris, 1989). In summary, we would suggest that our results are not a product of either the developmental age of the animals or the *in vitro* methods used.

Our electrophysiological data fit very well with observations on the distribution of the NK_1 receptor in the lumbar spinal cord, made using an antibody raised against the C-terminal sequence of the receptor. Similar results have also been reported using an antibody to another peptide sequence from the rat NK_1 receptor (Moussaoui et al., 1992). Contrary to expectations, the SG (lamina II) was found to be almost devoid of cells expressing NK_1 receptors whereas, neurons in lamina I or deeper neurons, particularly those in laminae III–IV frequently expressed the receptor. Other studies have demonstrated substance P–immunoreactive terminals on the dendrites of lamina I, spinothalamic neurons (Priestley and Cuello, 1989) and on those of intracellularly filled deeper dorsal horn neurons (De Koninck et al., 1992). In the recent study of De Koninck et al. (1992) in the cat, three wide dynamic range neurons in laminae III–IV were intracellularly labeled with horseradish peroxidase, and subsequently these neurons were shown to have many substance P containing synaptic contacts upon them. Interestingly, these neurons had dendrites that in some cases extended into lamina I, had few spines and were not postsynaptic to glomerular type synapses. Because of the dense plexus of substance P containing axons in lamina II it had always been assumed that many would terminate directly onto the small, intrinsic SG neurons. However, this study suggests that all the substance P containing primary afferents bypass lamina II neurons and terminate on neurons whose soma are outside this lamina. A similar conclusion has been proposed by Yoshimura et al. (1993) as a result of their findings with substance P application to SG neurons in the adult rat spinal cord slice. In an earlier study in the cat, Duggan et al. (1979) reported that microiontophoretic application of substance P in the SG had no effect on the responses of deeper-lamina nociceptive neurons. However, although this appears to concur with the present data it is somewhat surprising as the substance P should have acted on NK_1 receptors on dendrites traversing the SG.

In agreement with several other studies we do find that lamina II neurons respond to primary afferent stimulation at intensities that activate Aδ- and C-fibers with EPSPs. The latency of some of these responses would be compatible with their monosynaptic mediation. The origin of these responses has not been definitely established. It is possible that lamina II neurons do receive synaptic input from afferents containing both excitatory amino acid and substance P but only express receptors for the former. In a study, in the cat, in which SG neurons were intracellularly labelled, some synaptic profiles that contained both round clear vesicles and dense core vesicles were observed to synapse on dendrites of these cells (Gobel, 1980). However, several other synapses, such as those containing opioids have this ultrastructural appearance. The majority of substance P–immunoreactive synapses have been shown to terminate presynaptically to neurons expressing NK_1 receptors (Liu et al., 1993, 1994). However, there are also many terminals containing substance P that terminate in a pattern that suggests release at sites some distance from processes bearing these receptors, and thus this peptide may act through a process of volume transmission. This could explain some of the apparent lack of correlation between the immunocytochemical distribution of substance P and the distribution of the NK_1 receptor. The distribution of NK_1 receptors on neurons also suggests that they are not clustered in a subsynaptic site and that the majority of the cell surface would be available for the action of the peptide agonist (Liu et al., 1994). Interestingly, antibody microprobe studies have suggested that NKA may diffuse considerable distances through the dorsal horn from its primary afferent site of release (Duggan et al., 1990). The determination of how far from the primary afferents, tachykinins could exert their action would be determined by the activity and distribution of peptidases able to cleave these peptides (Duggan et al., 1992). It has been suggested that peptides such as CGRP and somatostatin may facilitate the action of substance P by competing as substrates for an endopeptidase that also degrades substance P (Le Greves et al., 1985). Overall, these data support the view that primary afferents containing substance P have as their target, cells other than those in lamina II.

It is more probable that lamina II neurons get a primary afferent monosynaptic input from "nonpeptide" (Hunt and Rossi 1985) afferents. Using both histochemistry for fluoride resistant acid phosphatase (FRAP) or lectin histochemistry a "nonpeptide" population of C-fiber primary afferents has been well documented (see Knyihar-Csillik and Csillik, 1981; Hunt and Rossi, 1985; Silverman and Kruger, 1988). These afferents could account for the excitation of SG neurons by small-diameter primary afferents; however, we are not aware of any direct study proving that these afferents terminate on neurons intrinsic to the SG.

Although the present data suggest that views of the organization of function within the SG needs considerable revision some reservations have to be expressed. To date only the NK_1 receptor has been studied in any detail and data is now required on the other tachykinin receptors and on receptors for other primary afferent peptides. As more data accumulates on the plasticity of the dorsal horn in inflammatory or neuropathic pain, it is clear that considerable potential for alterations in receptor expression and synaptic efficacy exist. Indeed, in some neuropathic conditions substantial rewiring could take place. Accepting these reservations, the operation of the small-diameter primary afferent system in acute pain probably needs a

major revision. Most models of the SG suggest that these neurons would act as relays between C-fiber inputs and projection neurons in lamina I and deeper-lamina neurons. Clearly, much of the input through peptidergic afferents could be directly to projection neurons in lamina I and deeper laminae. The precise function(s) of the SG will require much more detailed knowledge of its connections. Certainly from the wide range of excitatory and inhibitory neurotransmitters that are present in these neurons suggest that their operation is diverse. Some insight into the complexities involved can be found in the recent article of Randic et al. (1993).

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