The Journal of Neuroscience, February 1995, 15(2): 1363–1371

Synaptic Interactions between Primary Afferent Terminals and GABA and Nitric Oxide–Synthesizing Neurons in Superficial Laminae of the Rat Spinal Cord

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The superficial laminae (I and II) of the spinal dorsal horn receive small caliber primary afferent fibers responsive to noxious stimulation, and contain local circuit neurons that modulate afferent input. Many of these neurons are GABAergic; about a third of these also synthesize nitric oxide. We identified three main morphological types of primary afferent terminals in superficial laminae after injections of a tracer selective for small caliber afferents into the sciatic nerve of rats. The relative densities of the three types varied through the dorsoventral extent of laminae I and II. Synaptic contacts of each type with GABA- and nitric oxide synthase (NOS)-containing dendrites and axon terminals were determined by preembedding and postembedding immunocytochemistry.

Nonglomerular primary afferent terminals, likely to originate from peptidergic unmyelinated fibers, were not seen in synaptic contact with either GABA- or NOS-containing neurons. Primary afferent terminals at the center of type 1 glomeruli (C1) and at the center of type 2 glomeruli (C2) are likely to originate from unmyelinated and small myelinated fibers, respectively. GABAergic terminals contacted more C2 than C1 terminals, suggesting more effective presynaptic inhibition of C2 terminals. Many GABAergic terminals were also positive for NOS, but all GABAergic terminals presynaptic to primary afferent terminals were negative for NOS. Only C2 terminals established frequent synapses with NOS-positive dendrites. These results provide morphological evidence for selective inhibitory gating of input to superficial dorsal horn, and suggest that the link between noxious input and nitric oxide-synthesizing neurons, likely to be involved in nociception, may be provided both by direct synaptic contacts of small myelinated fibers onto dorsally extending NOS-positive dendrites, and by unmyelinated fibers via an oligosynaptic pathway.

[Key words: somatosensation, substantia gelatinosa, presynaptic inhibition, synaptic glomeruli, modulation of nociception]

An extensive literature deals with the morphological and functional properties of primary afferent fibers and their spinal terminations. Special attention has been paid to those afferents terminating in superficial laminae of the dorsal horn because of their involvement in the mediation of pain (Light, 1992). Primary afferents terminating in the superficial dorsal horn that mediate nociceptive input are likely to release glutamate as neurotransmitter (Schneider and Perl, 1985; Schouenborg and Sjölund, 1986; DeBiasi and Rustioni, 1988; Skilling et al., 1988; Yoshimura and Jessell, 1990). These fibers may also release other mediators, including aspartate and neuropeptides (Tracey et al., 1991; Kangrga and Randic, 1991; Light, 1992; Sorkin and McAdoo, 1993).

By combining tracing methods with electron microscopic postembedding immunocytochemistry, we have recently demonstrated enrichment of glutamate and aspartate in different types of primary afferent terminals (PAT) in the superficial laminae of the dorsal horn (Valtschanoff et al., 1994). These include the central (C) terminals of type 1 and type 2 glomeruli, referred to as C1 and C2, respectively (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva, 1994). Both types predominate in the inner part of lamina II; C1 terminals may contain dense core vesicles, seldom found in C2. C1 terminals stain more densely for glutamate and aspartate than C2 or a third type, also identified as originating from primary afferents in previous literature (Knyihár-Csillik et al., 1982a; Valtschanoff et al., 1992a; 1994). This type of terminal, which is not involved in glomerular arrangements, predominates in laminae I and outer II and displays numerous dense core vesicles.

We determine here the relations of PAT to GABAergic profiles in superficial laminae in order to explore mechanisms of central modulation of these afferents. Recent work has demonstrated the presence of neurons in the superficial laminae of the dorsal horn that may synthesize nitric oxide (NO, Valtschanoff et al., 1992b; Dun et al., 1993); most such neurons are GABAergic (Valtschanoff et al., 1992c; Spike et al., 1993). The involvement of NO in dorsal horn mechanisms responsible for nociception and hyperalgesia seems likely (Meller and Gebhart, 1993). Therefore, in this study we also investigate the relations of the different types of PAT in the superficial dorsal horn with neurons containing NO synthase (NOS).

Received Dec. 1, 1993; revised Aug. 5, 1994; accepted Aug. 10, 1994.

We thank K. D. Phend for her expert histological assistance. This work was supported by NINDS Grant 12440 to A.R. and by a fellowship from the Italian Ministry for the University and Scientific Research (Murst 40%) to P.S.B.

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Materials and Methods

Observations were from seven male Sprague–Dawley rats (Charles River, 150–500 gm) anesthetized with sodium pentobarbital (40 mg/kg). The left sciatic nerve was injected above the knee with 2% WGA-HRP (Sigma) dissolved in 5% dimethyl sulfoxide through a 30G needle attached with thin polyethylene tubing to a Hamilton microsyringe. After 2–3 d, rats were reanesthetized and perfused intra-aortically with 100 ml of heparinized saline, followed by 500 ml of a freshly prepared mixture of 2.5% glutaraldehyde, 0.5% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). Lumbar segments associated with the sciatic nerve were removed, postfixed for 2 hr in the same fixative, and stored in PB. Fifty micrometer thick transverse sections were cut on a Vibratome and reacted according to a tetramethyl benzidine/tungstate (TMB/T) protocol to reveal peroxidase (Weinberg and Van Eyck, 1991). Sections from three rats with dense anterograde labeling (usually from L4) were further processed for either postembedding immunostaining for GABA, or preembedding immunostaining for NOS, or a combination of the two. To avoid confusion in the text, the term "stained" refers to immunopositive elements, whether revealed by diaminobenzidine (DAB, in material processed for preembedding immunocytochemistry), or by gold particles (in postembedding immunocytochemistry), and the term "labeled" refers to the presence of the anterograde tracer.

For postembedding staining for GABA sections were immersed in 1% OsO_4 for 1 hr, stained in 1% uranyl acetate for 1 hr and wafer embedded in Epon-Spurr. For light microscopy, 1 μm thick sections were mounted on gelatin-coated slides and stained for GABA, and then were etched in potassium methoxide/propylene oxide for 2 min, pretreated in 0.3% hydrogen peroxide, incubated overnight in rabbit anti-GABA antibody (Sigma, 1:100 in phosphate-buffered saline, pH 7.5), then for 1 hr in biotinylated anti-rabbit antibody (Vector, 1:200) and for 1 hr in ExtrAvidin–peroxidase (Sigma 1:5000). For electron microscopy, small pieces of tissue were excised from the central portion of laminae I–III of L4 where the bulk of sciatic afferents to superficial laminae terminate (LaMotte et al., 1991) and glued to a plastic block for ultramicrotomy. Thin sections were mounted on uncoated nickel mesh grids and processed as described by Phend et al. (1992), as follows. Grids were washed with Tris-buffered saline containing 0.1% Triton X-100 (TBS+T), pH 7.6, incubated overnight in the primary antibody (1:10,000), rinsed in TBS+T, pH 7.6, transferred in TBS+T, pH 8.2, incubated in secondary antibody for 1 hr, rinsed, and dried. Goat anti-rabbit IgG conjugated to 20 nm or 30 nm gold particles (E-Y Labs, 1:25 in TBS+T, pH 8.2) was used as secondary antibody. Grids were counterstained with 5% uranyl acetate and Sato's lead and examined on a JEOL 200CX electron microscope. Preabsorption of the anti-GABA serum with free GABA blocked the postembedding staining.

For preembedding staining for NOS, sections pretreated in 1% sodium borohydride for 30 min were permeabilized with 50% ethanol for 10 min, incubated in 3% hydrogen peroxide (to inactivate endogenous peroxidases), and then in 10% normal goat serum (to block secondary antibody binding sites), and transferred into anti-NOS antibody (#6761-8, 1:5000) for overnight incubation at room temperature. Sections were then incubated 1 hr in biotinylated anti-rabbit antibody (Vector, 1:200) and 1 hr in ExtrAvidin–peroxidase complex (1:5000). Binding sites were visualized with DAB. The antibody for NOS was characterized in Schmidt et al. (1992). In the present material we verified that (1) NOS immunoreactivity coexisted, at light microscopic level, with histochemistry for NADPH diaphorase, a widely-accepted marker for NOS, and (2) NOS immunoreactivity gradually disappeared at progressively higher dilutions of the primary antiserum.

Camera lucida drawings from 1 μm thick sections stained with thionin supplied reference landmarks used to identify laminar boundaries in thin sections. Quantitative data were collected on the screen of the electron microscope (12,000×) or from prints enlarged from 20,000× negatives. Details on the samples employed for quantitative data are given with the description of the results.

Results

Morphology and distribution of labeled PAT

At the light microscope, the anterograde labeling was most prominent in lamina II, as expected from previous observations. This lamina consists of an outer cell-dense lamina IIo, and an inner cell-poor lamina IIi. In the cervical cord of rats, Ribeiro-da-Silva and Coimbra (1982) subdivided lamina II into three sublaminae: IIA (20 μm), IIBd (dorsal 20 μm), and IIBv (ventral, 45 μm). In the lumbar cord, lamina IIo corresponds to lamina IIA, and lamina IIi to lamina IIBd and IIBv. Labeling in our material was mainly in a band of the spinal gray identified, by the absence of myelinated fibers and the sparse cellular density, as IIi; labeling was densest in its dorsal portion. Laminae I and IIo contained sparse labeling, and the gray matter ventral to lamina II appeared virtually free of anterograde tracer (Fig. 1*A*).

At the electron microscope, labeled axons and their terminals were identified by the presence of amorphous deposits or rectangular crystals of TMB/T reaction product. Only terminals in which the reaction product did not obscure the main cytological features were included for quantitative analysis. We identified three morphological kinds of PAT: (1) sinuous terminals (C1) with dark axoplasm, sparse mitochondria and vesicles of variable size (Fig. 1*B*), sometimes including dense core vesicles (Fig. 1*C*), forming the central element of synaptic glomeruli of type 1 (Ribeiro-da-Silva and Coimbra, 1982); (2) scalloped terminals (C2) with light axoplasm, loosely packed uniform clear vesicles and several mitochondria, in some cases containing neurofilaments (Fig. 1*D,E*), and forming the central element of synaptic glomeruli of type 2 (Ribeiro-da-Silva and Coimbra, 1982); (3) terminals, mostly dome-shaped, with light axoplasm containing both clear and dense core vesicles, not involved in glomerular arrangements (Fig. 1*F*).

That the three kinds of terminals are not uniformly distributed in the superficial lamina has been reported in material without tracer labeling (Ribeiro-da-Silva, 1994). Out of 178 tallied labeled terminals, the majority were C1s (mainly in IIi); about 10% were C2s (exclusively in IIi), and about 25% were nonglomerular PAT (in I and IIo). Accurate estimates of terminal distribution are complicated by stereological considerations: smaller boutons, especially nonglomerular ones, are likely to be underrepresented. Also, not all terminals with morphological features of PAT were labeled in the plane of the section. This is in part to be expected as terminals of saphenous nerve are intermingled with those from the sciatic nerve in the central portions of the superficial laminae of L4 (LaMotte et al., 1991). However, in several sections from a rat in which C1 and C2 terminals were identified by their characteristic morphology, about 70% of C1 and only about 15% of C2 terminals were labeled. The percentage of nonglomerular PAT labeled was not determined, as unlabeled terminals of this type are likely to include those from intrinsic peptidergic neurons (Ribeiro-da-Silva et al., 1991).

GABA-positive profiles

Light microscopic postembedding immunocytochemistry for GABA confirmed previous observations demonstrating GABAergic neurons and terminals in lamina II of the dorsal horn (Fig. 2*A*; Barber et al., 1978; Todd and Spike, 1993). At the electron microscope, gold particles coding for GABA were enriched over some perikarya, dendrites, and axon terminals. In terminals they were dense over mitochondria and clear synaptic vesicles, though not all vesicles were overlaid by particles. GABA-stained terminals were small and dome-shaped with many flat or round clear synaptic vesicles, few mitochondria, and occasional dense core vesicles (Fig. 2*B–F*). They made single synaptic contacts of the symmetric type onto perikarya, dendrites, and axon terminals. Many GABA-positive dendrites contained vesicles. Vesicle-containing dendrites were small; their vesicles

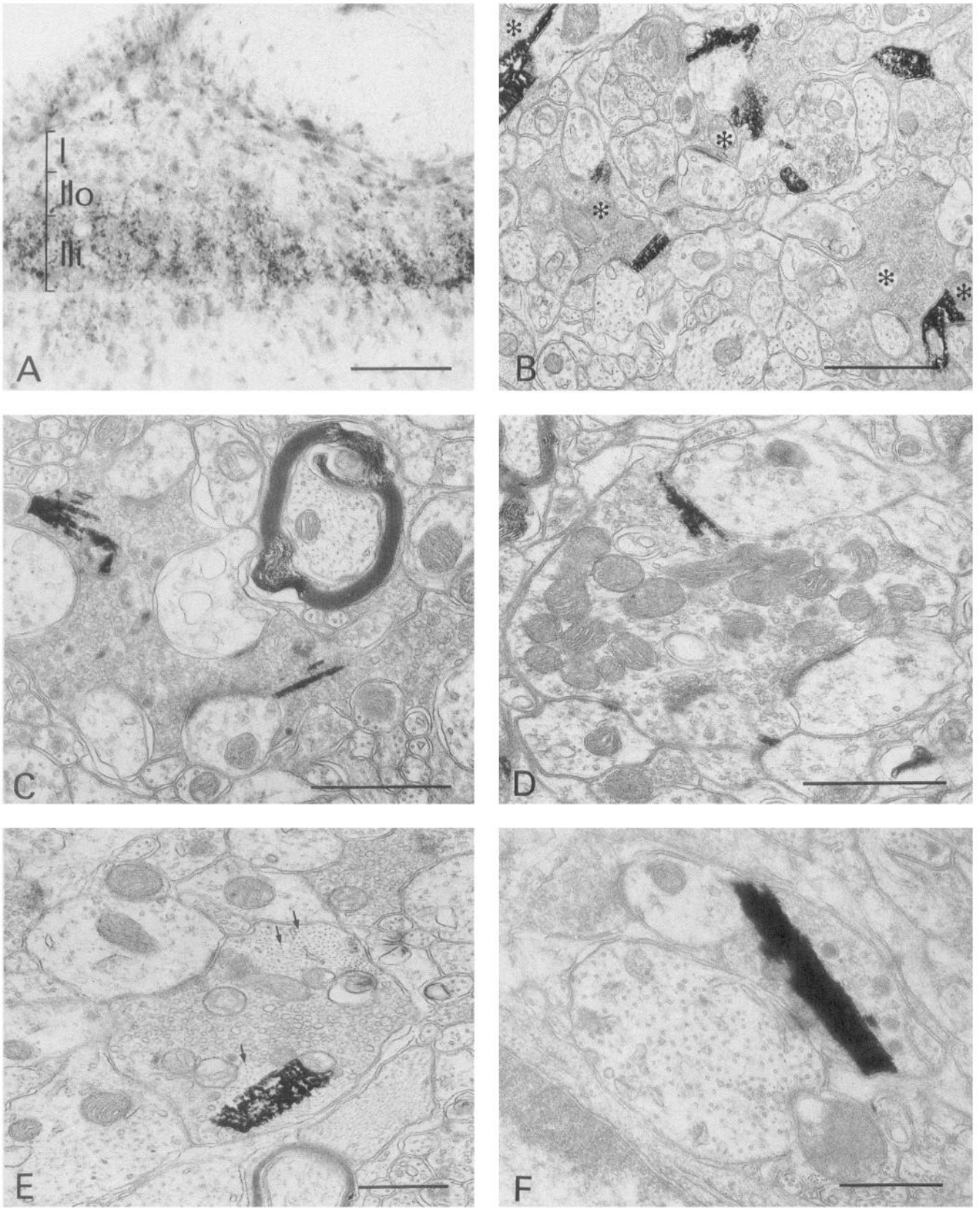

Figure 1. Anterograde labeling in superficial laminae of the dorsal horn after sciatic injection of WGA-HRP. *A,* Low-power photomicrograph shows that labeling is concentrated in the inner part of lamina II (*IIi*) at L4. Laminar boundaries as defined in the text. *B–E,* Electron microscopy of different types of labeled primary afferent terminals. *B,* Dark sinuous (C1) terminals (*asterisks*) in lamina II at L4, four labeled and one unlabeled. Terminals lacking dense core vesicles are the most frequently labeled among C1s. *C,* Labeled C1 terminal containing dense core vesicles. *D,* Labeled light scalloped (C2) terminal, containing many mitochondria, at the center of a type 2 glomerulus; this C2 terminal does not display neurofilaments in the plane of the section. *E,* Labeled C2 terminal containing neurofilaments (*arrows*). *F,* Small nonglomerular terminal in lamina IIo containing many dense core vesicles. Scale bars: *A,* 50 μm; *B-D,* 1 μm; *D* and *F,* 0.5 μm.

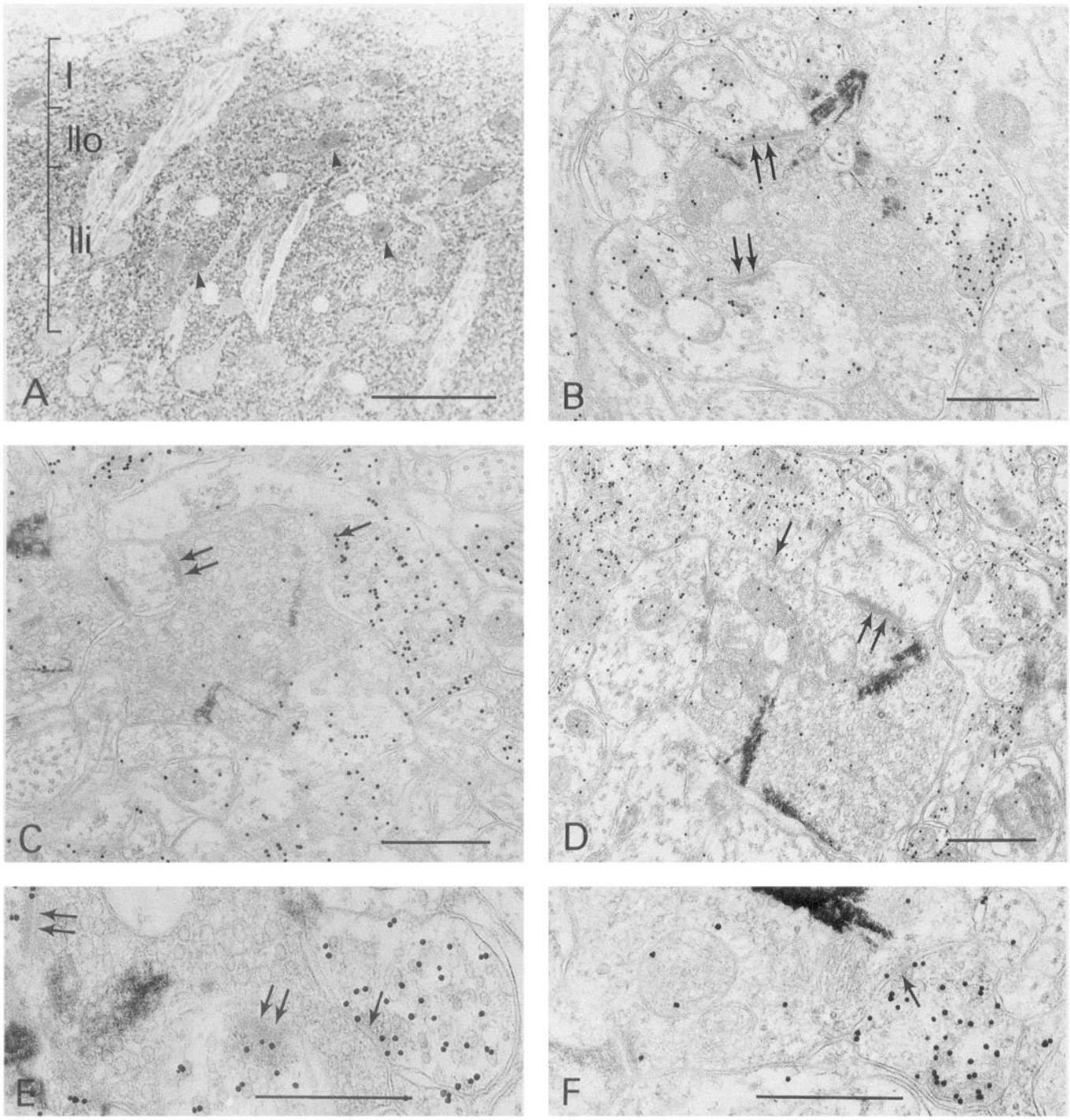

Figure 2. Immunostaining for GABA in superficial laminae. In *A* postembedding light microscopic staining was performed on 1 μm thick plastic section; examples of stained perikarya are indicated by arrowheads. In *B–F*, electron microscopic postembedding staining for GABA is combined with anterograde labeling of primary afferent terminals. *Double arrows* indicate synapses made by primary afferent terminals upon postsynaptic profiles. *Single arrows* indicate synapses made by GABA-positive endings upon primary afferent terminals. *B*, A C1 terminal without dense core vesicles is presynaptic to two dendrites enriched in gold particles. *C* and *D* show C1 (*C*) and C2 (*D*) primary afferent terminals postsynaptic to GABA-positive endings. *E* and *F*, Higher-power photomicrographs to show synapses of GABA-positive endings upon C1 (*E*) and C2 (*F*) terminals. Scale bars: *A*, 50 μm; *B–F*, 0.5 μm.

were relatively few and clustered at synaptic sites, rather than numerous and widespread, as in axon terminals (Fig. 2*D*).

Density of gold particles in GABA-positive profiles was generally highest over axon terminals, and higher over vesicle-containing dendrites than other dendrites. Vesicle-containing profiles that were enriched in GABA could be identified by their higher density of gold particles compared to other profiles (see Materials and Methods; Phend et al., 1992; Valtschanoff et al., 1994). For quantitative analysis, immunopositivity for GABA was defined as density of gold particles at least three times higher

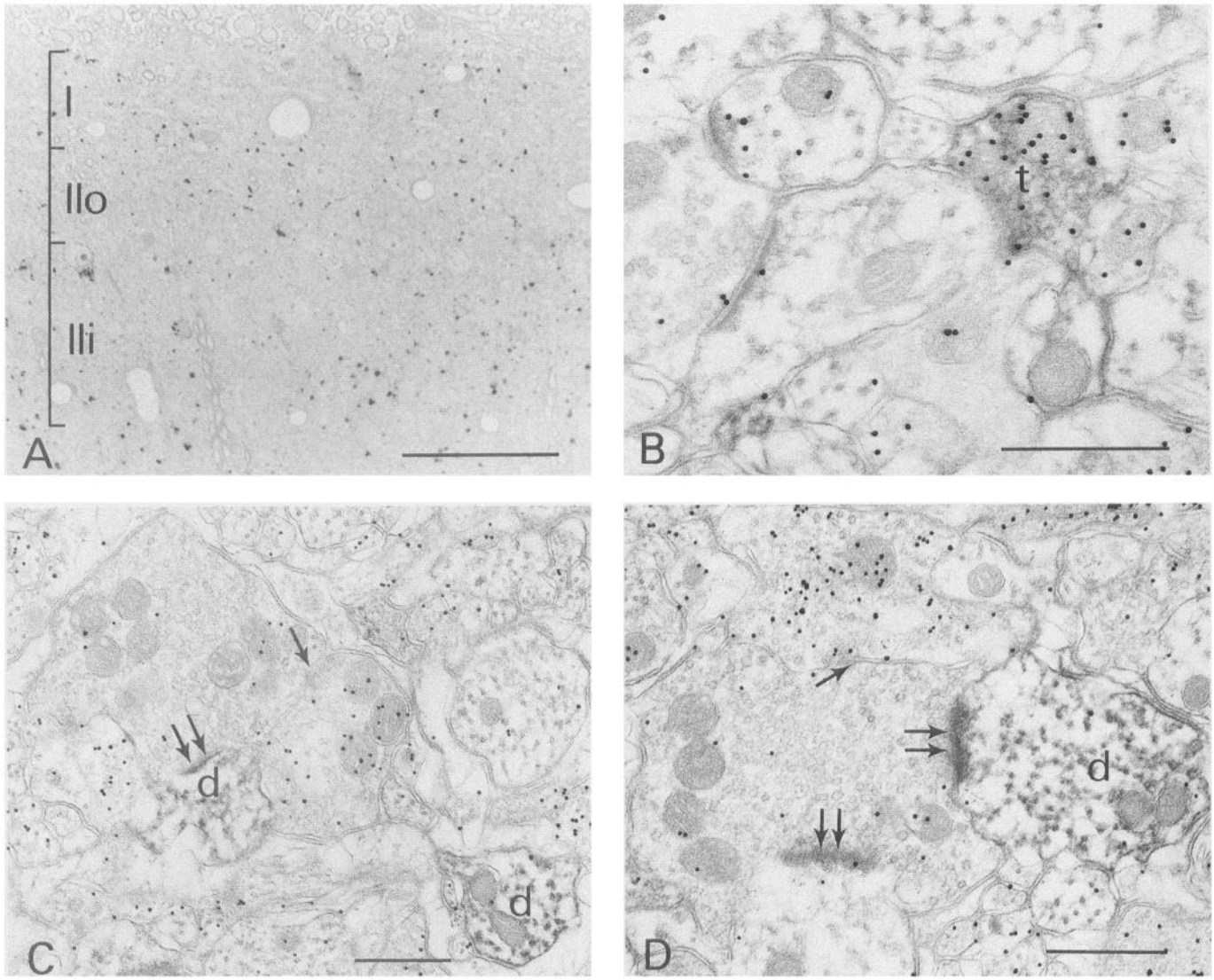

Figure 3. Immunostaining for NOS in superficial laminae. In *A*, light photomicrograph of a 1 μm thick plastic section from a 50 μm thick section processed for preembedding staining for NOS; staining consists mainly of *dark dots* corresponding to dendrites, axons, and terminals; *arrows* as in Figure 2. In *B–D*, Staining for NOS is combined with postembedding staining for GABA. *B*, An NOS-positive terminal (*t*) is also GABA-positive and contacts a negative dendrite. *C*, A C2 terminal contacts an NOS-positive dendrite (*d*); the terminal receives a synaptic contact from a GABA-positive ending. *D*, Another C2 terminal contacts an NOS-positive dendrite (*d*); a GABA-positive ending is presynaptic to the C2 terminal. Scale bars: *A*, 50 μm; *B–D*, 0.5 μm.

than in adjacent profiles (Valtschanoff et al., 1994). None of the PAT, whether identified by anterograde labeling or by morphological characteristics, were stained for GABA.

GABA-positive profiles were often in contact with PAT. The relation of GABA-positive profiles to PAT differed according to the type, as shown by the quantitative data of Table 1. C1 terminals were more frequently presynaptic to GABA-positive profiles than C2 (Fig. 2*C–F*). Of 15 C1 terminals presynaptic to GABA-positive profiles in this data set, 3 were presynaptic to conventional dendrites (Fig. 2*C*), 11 to vesicle-containing dendrites (Fig. 2*B*) and 1 contacted one of each kind. Of the seven C2 terminals presynaptic to GABA-positive profiles, five were presynaptic to conventional dendrites, and two to vesicle-containing dendrites. Some of the GABA-positive profiles postsynaptic to PAT were also presynaptic to unstained dendrites (Fig. 2*D*). C1 terminals were much less likely than C2 to be postsynaptic to GABA-positive profiles, generally axon terminals (Fig. 2*C,E*). In only one case was a C1 postsynaptic to a GABA-positive profile recognizable as a vesicle-containing dendrite.

Table 1. PAT and GABA-positive profiles

Type of PAT	*n*	Presynaptic to GABA	Postsynaptic to GABA
C1	54	15 (28%)	3 (6%)
C2	42	7 (17%)	9 (21%)

Data are from 96 photomicrographs, taken from superficial laminae in two rats; percentages from the total number of tallied PAT are in parentheses. PAT types are according to morphological criteria given in the text; C1 included 42 anterogradely labeled and 12 unlabeled terminals, and C2 included 6 labeled and 36 unlabeled. The majority of PAT were in nonsynaptic contact with or did not contact GABA-positive profiles. No contacts between nonglomerular terminals and GABA-positive profiles were seen.

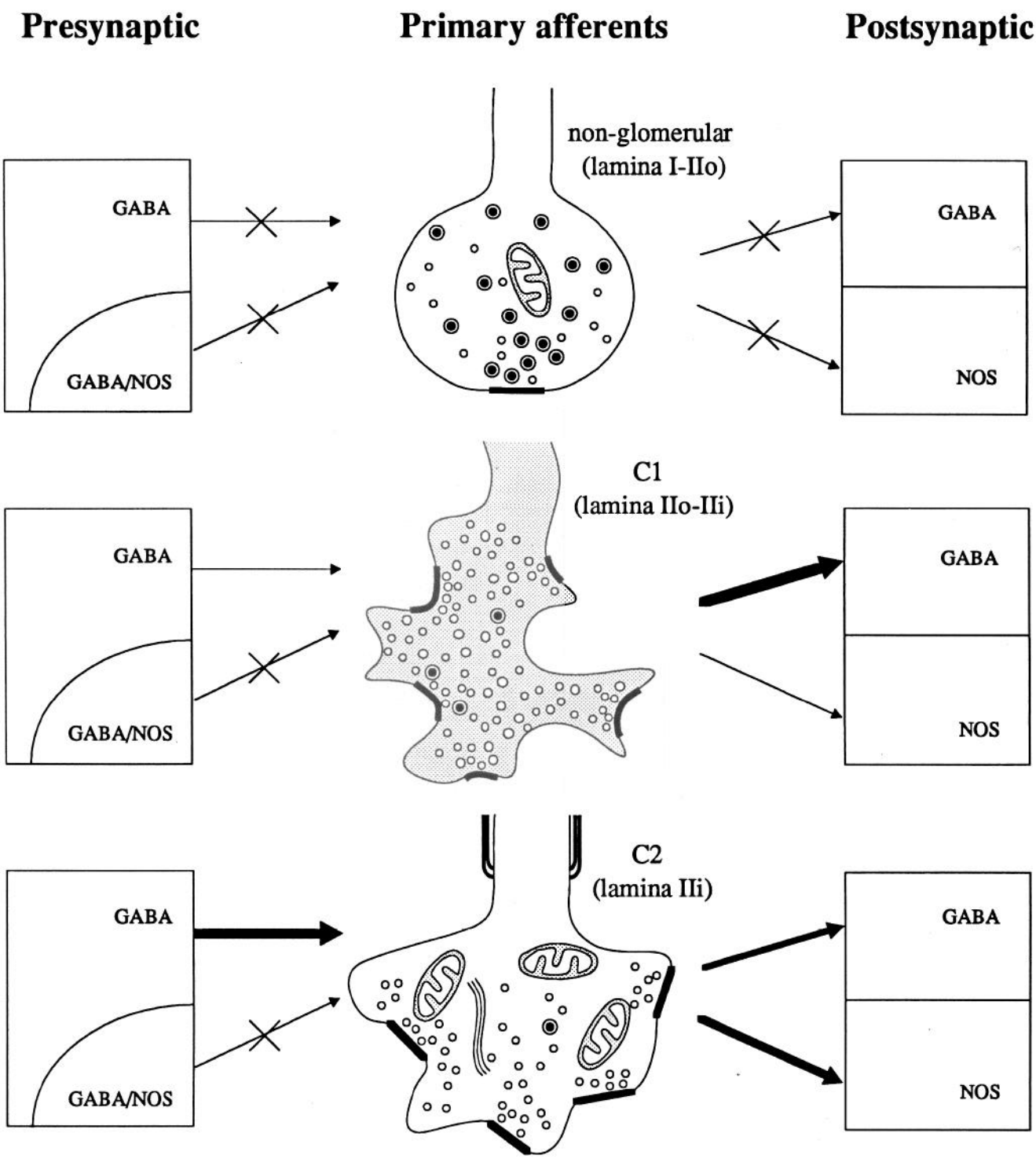

Figure 4. Summary and schematic representation of the results discussed in the text.

NOS-positive profiles

The laminar distribution of NOS immunoreactivity was as reported previously (Valtschanoff et al., 1992b; Dun et al., 1993; Fig. 3*A*). At the electron microscope it was present in perikarya, dendrites, myelinated and unmyelinated axons, and synaptic terminals. NOS-positive perikarya were small, with deeply indented nuclei and a narrow ring of cytoplasm. Electron-dense reaction product was associated with membranes; neurofilaments and microtubules of immunopositive axons and dendrites were also stained. Staining was not observed in endothelial cells or astrocytes. NOS-positive terminals were small, with few mitochondria and symmetric active zones. They contained a uniform population of clear vesicles and occasional dense core vesicles (Fig. 3*B*). Double staining revealed frequent colocalization of NOS with GABA as previously demonstrated (Fig.

3B; Valtschanoff et al., 1992c). However, many GABA-positive profiles were NOS-negative, and only 8 of 112 randomly selected NOS-positive dendrites were noticeably enriched in GABA.

None of the PAT exhibited NOS immunopositivity. Axodendritic but not axoaxonic synaptic contacts were seen between PAT and NOS-positive profiles. Contacts between C1 terminals and NOS-positive dendrites were very sparse and no relations existed between nonglomerular boutons and NOS-positive dendrites. Contacts onto NOS-positive dendrites were commonly seen with C2 terminals (Fig. 3C,D). Counts were made from two animals from the region (IIi-dorsal III) where the highest density of NOS-positive profiles was found: out of a total of 35 C2 terminals, 9 (26%) contacted NOS-positive dendrites.

The relations of three types of PAT with GABAergic and NOS-positive profiles are summarized in Figure 4.

Discussion

Types of terminals labeled by WGA-HRP

For identification of PAT we used the transport of WGA-HRP injected in the sciatic nerve. Our observations were therefore limited to terminals in the superficial laminae of the lumbar enlargement, presumably endings of unmyelinated and small myelinated primary afferent fibers. A selectivity of WGA-HRP for these fibers has been reported previously and related to the affinity of this tracer for *N*-acetyl-D-glucosamine and neuraminic acid (Robertson and Grant, 1985; Valtschanoff et al., 1992a). While it is generally agreed that WGA-HRP is taken up by unmyelinated fibers in the peripheral nerve, small myelinated fibers may also take up the tracer to a variable extent (depending on concentration, survival time, solvent, etc.). We observed labeling only in unmyelinated fibers in transverse sections of L4 dorsal root 2–3 d after injection of the sciatic nerve (Valtschanoff et al., 1992a). Given the predominance of labeled C1 terminals, likely to be endings of unmyelinated fibers (Ribeiro-da-Silva and Coimbra, 1984; Ribeiro-da-Silva, 1994), the present results confirm selectivity of WGA-HRP for these fibers in the PNS. However, the labeling of some C2 terminals, perhaps arising from small myelinated primary afferents (Coimbra et al., 1984), supports previous indications that the selectivity may not be absolute (LaMotte et al., 1991). Nonglomerular terminals, predominating in lamina I and IIo and characterized by the presence of dense core vesicles, are likely to include peptidergic endings of unmyelinated primary afferents; it is, therefore, not surprising that they were labeled by WGA-HRP.

The identification of three main classes of PAT is a foundation for further investigation on synaptic mechanisms in superficial laminae. These three classes of terminals have been identified in superficial laminae of the rat cervical cord (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva et al., 1985; Cruz et al., 1993), and resemble those identified in cats and monkeys (Knyihár-Csillik et al., 1982a; Maxwell and Réthelyi, 1987; Carlton and Hayes, 1990; Hayes and Carlton, 1992) though their contacts with adjacent dendrites and axon terminals may be more elaborate in these species than in rats. The relative laminar distribution of the three types reported here is generally consistent with that in the cervical cord of rats (Ribeiro-da-Silva and Coimbra, 1982) and the lumbar cord of monkeys (Knyihár-Csillik et al., 1982a). Most PAT in lamina I were nonglomerular; moving ventrally these terminals were gradually replaced by C1s. C1 and C2 terminals were intermingled in IIi, with C1 decreasing and C2 increasing from dorsal to ventral within this sublamina (see also Ribeiro-da-Silva, 1994). Nonglomerular and some C1 terminals contained dense core in agreement with the high density of immunostaining for peptides in laminae I and IIo of the rat lumbosacral cord (Light, 1992).

Synaptic relations to GABAergic neurons

The present results show that the three types of terminals have different synaptic relations with GABAergic neurons. C1 terminals contacted more GABAergic dendrites than did C2 terminals, whereas C2 terminals were more likely than C1 terminals to be postsynaptic to GABAergic processes. Since C1 terminals contacted a higher fraction of vesicle-containing dendrites, more likely than other dendrites to be identified as GABA-positive, it is possible that C1 and C2 terminals contact GABAergic dendrites in comparable numbers. Given the large size of these primary afferent terminals, stereological considerations suggest that all terminals of both C1 and C2 may contact GABAergic dendrites, and that all C2 terminals may be postsynaptic to a GABAergic terminal.

These results are consistent with previous morphological studies in rodents and primates showing that C2 terminals are more frequently postsynaptic to axon terminals than are C1 (Knyihár-Csillik et al., 1982b; Ribeiro-da-Silva et al., 1985; Hiura et al., 1991; Cruz et al., 1993). Terminals of different functional classes in the superficial laminae of the spinal cord of monkeys may also differ in their relations with presynaptic GABAergic profiles. Whereas boutons of Aδ (small myelinated) fibers were consistently postsynaptic to GABAergic axon terminals (Alvarez et al., 1992), none of the boutons of the two intracellularly filled C fibers examined were postsynaptic to such terminals (Alvarez et al., 1993). Comparison between these results and the present data are complicated by differences in sample collection (limited but functionally identified in monkeys, extensive but without functional identification in the present material) and species differences (e.g., glomeruli in monkeys are more complex than in rats). However, the present data and the studies reviewed above concur in suggesting that peptidergic unmyelinated (C) fibers giving rise to nonglomerular terminals in laminae I and IIo lack GABAergic modulation, whereas C1 terminals in IIi receive modest GABAergic modulation, and C2 terminals in IIi receive strong GABAergic modulation. This differential inhibitory modulation may be associated with the relatively poor spatiotemporal resolution characteristic of C fibers (Willis and Coggeshall, 1991). The present results suggest that, at least in rodents, presynaptic inhibition plays a less significant role in the spinal gating of nociception than is the case for other cutaneous modalities Based on recent evidence, GABA mechanisms modulating pain sensibility differ, compared to other cutaneous modalities, also at thalamic level (Ralston and Ralston, 1994).

Synaptic relations to NO-synthesizing neurons

GABAergic neurons in superficial laminae are not homogeneous. A complex pattern of colocalization of GABA with other putative neurotransmitters is emerging: many GABAergic neurons also contain glycine, ACh, and/or various peptides (Ribeiro-da-Silva and Cuello, 1990; Doyle and Maxwell, 1993; Spike et al., 1993). About one-third of GABAergic neurons in the superficial dorsal horn contain NOS, the enzyme responsible for the synthesis of NO (Valtschanoff et al., 1992c; Spike et al., 1993). These neurons may be involved in the mediation of nociception and hyperalgesia (Meller and Gebhart, 1993). Changes in synaptic efficacy associated with hyperalgesia may

share features in common with higher centers, as NOS coexists with GABA also in interneurons in hippocampus and cortex, where NO is implicated in long-term potentiation (Valtschanoff et al., 1993a,b).

NOS was not present in GABA-positive terminals presynaptic to PAT; thus GABA-positive neurons involved in presynaptic inhibition are unlikely to synthesize NO. C2 terminals commonly contacted NOS-positive dendrites, in contrast to the other two types. NO may be released from dendrites in a nonsynaptic manner, possibly diffusing a considerable distance to reach its target. Neuronal NOS is a calcium-dependent enzyme with slow kinetics (Nathan, 1992). Therefore, synapses onto NOS-positive dendrites may be able to raise calcium sufficiently to trigger NO release only during sustained intense activity. Since NOS-positive neurons are concentrated in IIi and dorsal lamina III, ventral to the major focus of noxious input (Light, 1992; Valtschanoff et al., 1992c), the link between noxious input and NO may be provided both by direct synaptic contacts of Aδ fibers onto dorsally extending NOS-positive dendrites, and by unmyelinated fibers via oligosynaptic pathways. In addition, peptides released from C1 and nonglomerular terminals can also increase intracellular calcium levels (Womack et al., 1988). During sustained noxious stimulation peptides may diffuse ventrally (Duggan et al., 1990) and act synergistically to trigger NO synthesis.

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