Laminar Compartmentalization of $GABA_A$-Receptor Subtypes in the Spinal Cord: An Immunohistochemical Study

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To assess the significance of $GABA_A$-receptor heterogeneity, which is based on a family of at least 15 subunits, the cellular localization and subunit composition of $GABA_A$-receptor subtypes were analyzed immunohistochemically in the rat spinal cord. The distribution of subunits $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\beta2,3$, and $\gamma2$ was investigated with subunit-specific antibodies, and their colocalization within individual neurons was visualized by double-immunofluorescence staining. The results reveal a widespread expression of the subunits $\alpha3$, $\beta2,3$, and $\gamma2$ in the spinal cord, whereas the three other α subunits displayed a more restricted, lamina-specific distribution. The $\alpha1$ and $\alpha5$ subunits were most abundant in the intermediate zone, whereas the $\alpha2$ subunit was predominant in the superficial layers of the dorsal horn and in somatic and preganglionic motoneurons. From colocalization studies, seven subunit combinations could be identified ($\alpha3/\beta2,3/\gamma2$; $\alpha2/\beta2,3/\gamma2$; $\alpha1/\beta2,3/\gamma2$; $\alpha5/\beta2,3/\gamma2$; $\alpha1/\alpha5/\beta2,3/\gamma2$; $\alpha2/\gamma2$; $\alpha2/\alpha5/\gamma2$) that correspond presumably to distinct receptor subtypes. Although most neurons expressed the subunit triplet $\alpha x/\beta2,3/\gamma2$, the $\beta2,3$ subunits could not be detected in motoneurons that may thus possess "atypical" receptor subtypes ($\alpha2/\gamma2$ and $\alpha2/\alpha5/\gamma2$). On the subcellular level, aggregates of immunoreactivity, suggestive of postsynaptic $GABA_A$ receptors, typically were seen on the surface of neuronal somata and proximal dendrites. In addition, an intense, diffuse staining was observed in laminae I–III for the subunits $\alpha2$, $\alpha3$, $\beta2,3$, and $\gamma2$, presumably localized on primary afferent terminals. The localization of $GABA_A$-receptor subtypes in distinct laminar compartments of the spinal cord suggests that $GABA_A$-receptor heterogeneity is of relevance for the modulation of sensory inputs, nociception, and motor control at segmental levels.

Key words: inhibitory neurotransmission; motoneurons; benzodiazepines; pain control; allodynia; receptor subtypes

For inhibitory neurotransmission in the spinal cord, glycinergic mechanisms have received much emphasis in the past. However, there is growing morphological, physiological, and pharmacological evidence that GABA is of major functional importance at segmental levels (Hunt, 1983; Nistri, 1983; Willcockson et al., 1984; Magoul et al., 1987; Schneider and Fyffe, 1992; Todd and Spike, 1993; Rogers et al., 1994). GABA is found in numerous local circuit neurons (Barber et al., 1982) as well as in bulbospinal projection neurons, which innervate principally the dorsal horn (Reichling and Basbaum, 1990; Holstege, 1991; Jones et al., 1991). Further, the GABAergic system plays a pre-eminent role in presynaptic inhibition of primary afferents (primary afferent depolarization), thus modulating sensory transmission, nociception, and motor activity on both pre- and postsynaptic levels (Eccles et al., 1963; Barker and Nicoll, 1972; Barber et al., 1978; Polc, 1982; Cattaert et al., 1991; Liu et al., 1992; Stuart et al., 1992). GABA and glycine are frequently colocalized within single terminals (Todd and Sullivan, 1990; Todd and Spike, 1993), and alterations in synaptic inhibition affecting either neurotransmitter system contribute to the development of chronic pain syndromes and hyperalgesia (Yaksh, 1989; Yamamoto and Yaksh, 1993; Sivilotti and Woolf, 1994; Sluka et al., 1993, 1994; Woolf and Doubell, 1994), although these mechanisms remain poorly understood.

Most of the synaptic inhibitory action of GABA is mediated by $GABA_A$ receptors, which constitute hetero-oligomeric chloride channels encoded by a family of at least 15 subunit genes ($\alpha1$–6, $\beta1$–3, $\gamma1$–3, δ, $\rho1$–2). This extensive structural heterogeneity gives rise to multiple receptor subtypes differing in subunit composition and functional properties (Wisden and Seeburg, 1992; Macdonald and Olsen, 1994; Mohler et al., 1995). To elucidate the significance of $GABA_A$-receptor subtypes for sensory–motor processing and pain perception at segmental levels, a detailed knowledge of their subunit composition and cellular distribution will be required. Only limited information is available on the expression of $GABA_A$-receptor subunits of the spinal cord, derived mostly from *in situ* hybridization studies (Persohn et al., 1991; Wisden et al., 1991; Ma et al., 1993). Thus, strong signals have been reported for the $\alpha2$-, $\alpha3$-, $\beta3$-, and $\gamma2$-subunit mRNA, whereas the other subunits were only weakly expressed ($\alpha1$, $\alpha5$, $\beta1$, $\beta2$, $\gamma1$, $\gamma3$) or not detected ($\alpha6$, δ). In addition, the $\alpha1$ and $\beta2,3$ subunits have been mapped immunohistochemically in the rat and human spinal cord (Waldvogel et al., 1990; Bohlhalter et al., 1994). It remains unknown, however, whether multiple $GABA_A$-receptor subtypes are present in the spinal cord and whether their expression correlates with defined neuronal circuits.

The aim of the present study was to analyze immunohistochemically the regional and cellular distribution of the $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\beta2,3$, and $\gamma2$ subunits in rat spinal cord, and to identify putative receptor subtypes based on the colocalization of subunits within individual neurons. These subunits were selected in preliminary experiments that demonstrated their presence in the

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spinal cord, whereas the α6 and δ subunits could not be detected. The well defined lamination of the spinal cord (Molander et al., 1984, 1989) served as a basis for allocating receptor subtypes to functionally distinct neuron populations, notably those involved in sensory processing and nociception in the dorsal horn.

MATERIALS AND METHODS

Antibodies. The $GABA_A$-receptor subunits α1, α2, α3, α5, and γ2 were visualized using subunit-specific antisera raised in rabbit (α1) and guinea pigs (α2, α3, α5, and γ2) against synthetic peptides sequences derived from the respective rat subunit cDNA [α1 subunit, N-terminal residues 1–16; α2 subunit, 1–9; α3 subunit, 1–15; α5 subunit, 1–10; γ2 subunit, 1–29; for antibody characterization, see Fritschy and Mohler (1995); Mohler et al. (1995), and references therein]. In addition, the mouse monoclonal antibody bd-17 (Schoch et al., 1985), recognizing both the β2 and β3 subunits (Ewert et al., 1990), was used.

Tissue preparation. Adult male Sprague–Dawley rats (Institut für Tierkunde, University of Zurich) weighing 150–200 gm were deeply anesthetized with chloral hydrate (400 mg/kg; i.p.) and perfused through the ascending aorta with a mixture of 2–4% *p*-formaldehyde and 15% of a saturated solution of picric acid in 0.15 M phosphate buffer, pH 7.4 (Fritschy and Mohler, 1995). The spinal cord was removed immediately after the perfusion and postfixed for 2–6 hr in the same fixative at 4°C. Thereafter, the tissue was processed with a modified antigen-retrieval protocol (Evers and Uylings, 1994; Shi et al., 1995) aimed at optimizing the signal-to-noise ratio in the subsequent immunohistochemical staining. Blocks of spinal cord were preincubated overnight at room temperature in 0.1 M sodium citrate buffer, pH 4.5, and irradiated with microwaves (95°C, 120 sec) at full power (650 W) in the same buffer. Thereafter, the tissue was stored for 3 hr in PBS containing 10% dimethyl sulfoxide for cryoprotection. Transverse and horizontal 40 μm sections from cervical, thoracic, and lumbar levels were cut from frozen blocks with a sliding microtome and collected in ice-cold 0.05 M Tris-saline buffer (TBS), pH 7.4.

Immunohistochemistry. The regional distribution of the $GABA_A$-receptor subunits was analyzed in sections processed for immunoperoxidase staining (Hsu et al., 1981). Free-floating sections were incubated for 3 hr at room temperature in primary antibodies diluted in TBS containing 10% normal serum and 0.2% Triton X-100. The following dilutions were used: α1 subunit, 1:20,000; α2 subunit, 1:10,000; α3 subunit, 1:6,000; α5 subunit, 1:4,000; γ2 subunit, 1:3,000; β2,3 subunits (bd-17), 1.4 μg/ml. The sections were then washed in TBS, and the staining procedure was performed according to the specifications of the manufacturer (Vectastain Elite kits; Vector Laboratories, Burlingame, CA) using diaminobenzidine hydrochloride (Sigma, St. Louis, MO) as chromogen. Sections were mounted on gelatin-coated slides, air-dried, dehydrated with an ascending series of ethanol, and coverslipped out of xylene. For specificity controls, primary antibodies were preincubated with their corresponding peptide antigen (0.5–5 μg/ml of antibody solution) for 3 hr at room temperature before being applied to the sections. This procedure resulted in a dose-dependent loss of specific staining.

The colocalization of subunits within individual neurons was assessed by double-immunofluorescence staining. Sections were incubated for 3 hr at room temperature in a mixture of two primary antibodies diluted in TBS containing 10% normal serum and 0.2% Triton X-100. The following nine combinations were tested, which reflect the availability of primary antisera raised in different species: α1/α2, α1/α3, α1/α5, α1/γ2, α1/β2,3, α2/β2,3, α3/β2,3, α5/β2,3, and γ2/β2,3. The concentration of primary antibodies was three to five times higher than for immunoperoxidase staining. After a wash in TBS, the sections were incubated for 1 hr with a combination of secondary antibodies coupled to either dichlorotriazinylaminofluorescein (DTAF; 1:100), Cy3, or Cy5 (1:200; Jackson Immunoresearch, West Grove, PA). The sections were then washed, rinsed quickly with distilled H_2O, mounted onto gelatin-coated slides, air-dried, and coverslipped with buffered glycerol.

Data analysis. The laminar distribution of $GABA_A$ subunits in the spinal cord was analyzed by light microscopy in sections processed for immunoperoxidase staining. Adjacent sections stained with Cresyl violet were used for cytoarchitectonic reference, according to the criteria established by Molander et al. (1984, 1989). Because the same pattern of subunit expression was observed at all levels of the spinal cord, the analysis of subunit colocalization was restricted to sections through the cervical enlargement. Sections processed for double-immunofluorescence staining were visualized by confocal laser microscopy (Leica, TCS 4D; Heidelberg, Germany) and processed on a Silicon Graphics workstation using the image analysis software IMARIS (Bitplane, Zurich, Switzerland). The extent of colocalization between the different subunits was analyzed semiquantitatively by counting the proportion of neurons immunoreactive for one subunit that also were positive for the other subunit examined (Table 1). For each subunit combination, the data are derived from 50–100 neurons per layer investigated.

RESULTS

Selectivity of the antisera

The subunit-specific antisera used in this study have been extensively characterized biochemically by Western blotting and immunoprecipitation (for review, see Mohler et al., 1995). Their suitability for immunohistochemistry has been documented in several previous reports (Benke et al., 1994; Bohlhalter et al., 1994; Fritschy et al., 1994; Fritschy and Mohler, 1995; Gao et al., 1995). In the present study, specificity controls included preabsorption of the antisera with their corresponding peptide, resulting in a dose-dependent loss of specific immunoreactivity (IR) (data not shown). Further, the pretreatment of the tissue by microwave irradiation in sodium citrate buffer (see Materials and Methods) produced a dramatic reduction in nonspecific staining, including staining of glial cells in the white matter that usually bind secondary antibodies nonspecifically.

Overview

At the macroscopical level, the IR for the $GABA_A$-receptor subunits analyzed (α1, α2, α3, α5, β2,3, and γ2) was widespread across the spinal cord, but exhibited marked differences in laminar distribution (Figs. 1–3). The staining was restricted to the gray matter, although numerous immunoreactive dendrites could be seen extending into the white matter, notably in the lateral and ventral funiculi. An intense labeling was observed in the superficial layers of the dorsal horn (laminae I–III) for the subunits α2, α3, β2,3, and γ2, whereas the α1 and α5 subunits were restricted to layer III. In the deep dorsal horn, intermediate zone, and most of the ventral horn (laminae IV–VIII), a moderate-to-strong staining was observed for the α1, α3, β2,3, and γ2 subunits, whereas the α2-subunit IR was moderate in layers IV–VI and weak in layers VII–VIII and the α5-subunit IR moderate throughout these layers. Somatic and preganglionic motoneurons (lamina IX and lateral cell column) exhibited a particularly intense staining for the α2 subunit and were stained moderately to strongly for the α5 and γ2 subunits. By contrast, the α1, α3, and, surprisingly, the β2,3 subunits were not detected in these cells. Hence, the subunits α3, β2,3, and γ2 were the most ubiquitous, whereas the other three α subunit variants were restricted to specific laminae (Figs. 1–3), suggesting the existence of multiple $GABA_A$-receptor subtypes in the spinal cord. No obvious difference in the laminar distribution or relative staining intensity

Table 1. Colocalization of the α subunit variants in spinal neurons

Lamina	Subunit combinations (%)				
	α1α2	α1α3	α1α5	α2α1	α5α1
III	20	0	60	15	15
IV–V	-	0	20	-	40
VI–VIII	-	0	40	-	30
IX	0	-	0	0	0

In each column, the values represent the percentage of neurons immunoreactive for the first subunit, which are also positive for the second subunit. Each combination was assessed on 50–100 neurons per layer. The layers in which one of the subunits was not sufficiently expressed for the combinatorial analysis are indicated by hyphens.

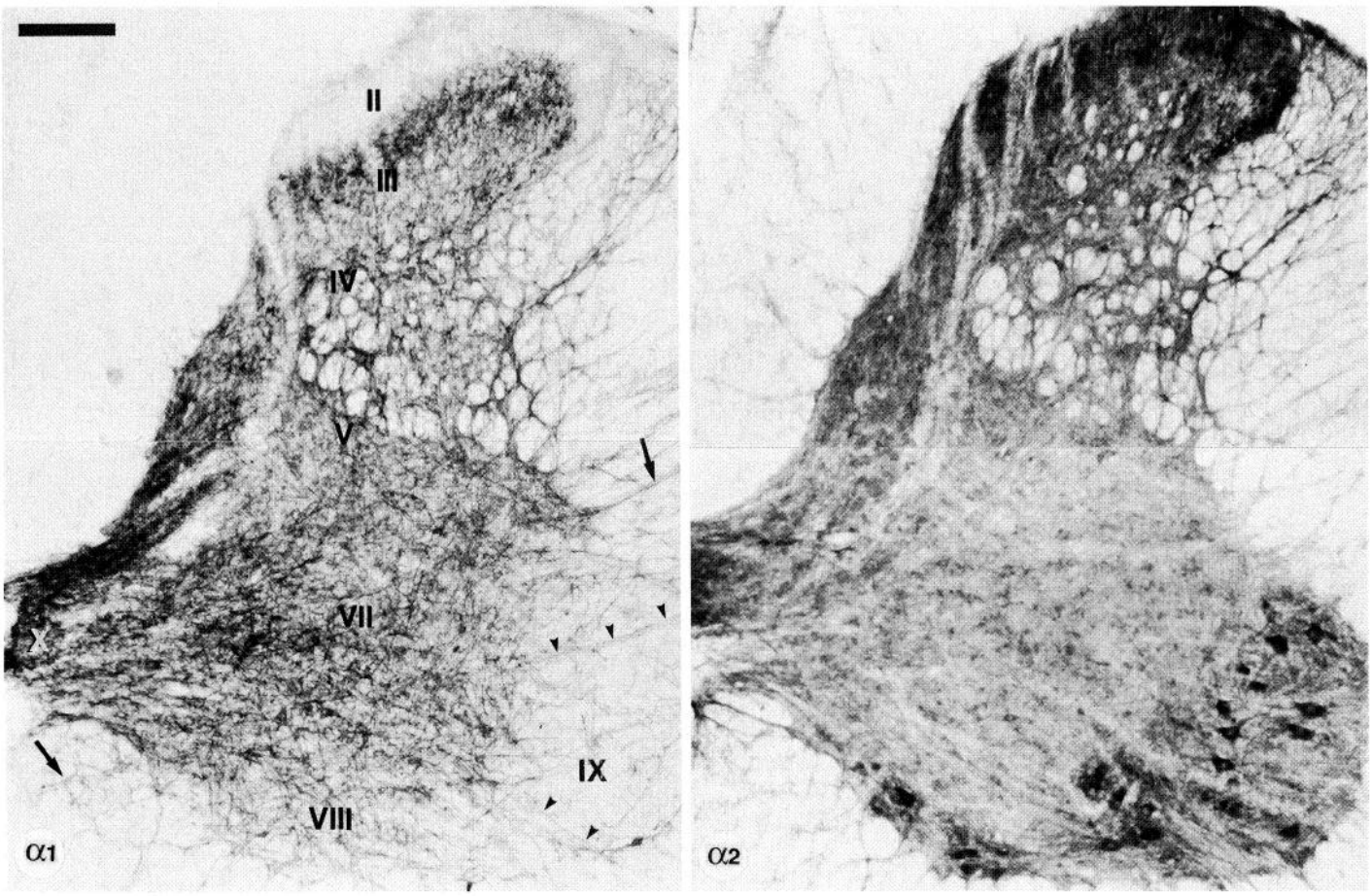

Figure 1. Complementary distribution of the $GABA_A$-receptor subunits α1 and α2 in the spinal cord, as seen in transverse sections through midcervical levels processed for immunoperoxidase staining with subunit-specific antisera. See Figures 2 and 3 for a comparison with the remaining subunits. An intense α2-subunit IR is seen in layers II and IX, which are devoid of α1-subunit staining. By contrast, layers V–VIII exhibit a prominent α1-subunit IR, and only a weak staining for the α2 subunit. *Arrowheads* point to α1-subunit-positive processes oriented in the plane of the section, and *arrows* indicate immunoreactive dendrites penetrating into the white matter in the ventral and lateral funiculi. Scale bar, 200 μm.

of subunits could be detected between cervical, thoracic, and lumbar levels of the spinal cord.

Laminar and cellular localization of $GABA_A$-receptor subunits

In the superficial laminae of the dorsal horn (layers I–III), all antibodies produced an intense staining, with a different pattern for each subunit (Figs. 1–4). The α1 and α5 subunits were unique for their absence of staining in layers I–II (Fig. 4*A*,*B*). This pattern contrasted with the intense and diffuse staining seen for the other subunits, in which cellular elements were difficult to recognize (Fig. 4*C*,*D*) even by confocal laser microscopy (see Fig. 9*A*). The α2-, β2,3-, and γ2-subunit IR was uniformly intense across lamina II, whereas the α3-subunit IR was stronger in the outer half of this layer (Fig. 4*C*,*D*). In lamina III, all subunits were detected, but with clear differences in their cellular distribution. For instance, the α1-subunit IR distinctly stained a population of small neurons in the outer half of this layer (Fig. 4*A*), whereas the α5 subunit was most abundant in the inner half of lamina III (Fig. 4*B*). In horizontal sections, these neurons appeared lenticularly shaped, with long dendrites running rostrocaudally within lamina III. The staining for the α3, β2,3, and γ2 subunits was strong and diffuse in lamina III, and individual immunoreactive neurons could be distinguished clearly only by confocal laser microscopy. In contrast, the α2-subunit IR was moderate in layer III, except for a few intensely stained cells (Fig. 4*C*).

Laminae IV and V generally were less intensely stained than the superficial layers of the dorsal horn. The α1 subunit, and to a lesser extent the α5-subunit antisera, labeled large multipolar cells, revealing their morphology in considerable detail. Many of these cells were seen to extend their dendrites dorsally into lamina III, as shown for α1-subunit staining (Fig. 4*A*). The α5 subunit labeled an additional population of smaller cells in lamina V that were apparently negative for the α1 subunit. The α3-, β2,3-, and γ2-subunit IR also was moderately strong in laminae IV and V, located primarily on small, inconspicuous cells. In addition, these subunits and the α2 subunit exhibited a diffuse staining similar to that seen in the superficial layers (Fig. 4*C*,*D*).

In laminae VI–VIII, the α3-, β2,3-, and γ2-subunit IR was the most widespread, labeling numerous, morphologically heterogeneous neurons. By contrast, the α1 and α5 subunits were restricted to subsets of neurons, for the most part large multipolar cells, with long dendrites extending in the transverse plane (Figs. 1–3, 5*C*), whereas the α2-subunit IR labeled only a few isolated neurons in these layers. Compared with the superficial layers, the staining was less diffuse and the morphology of immunoreactive neurons was revealed by the distribution of immunoreactive

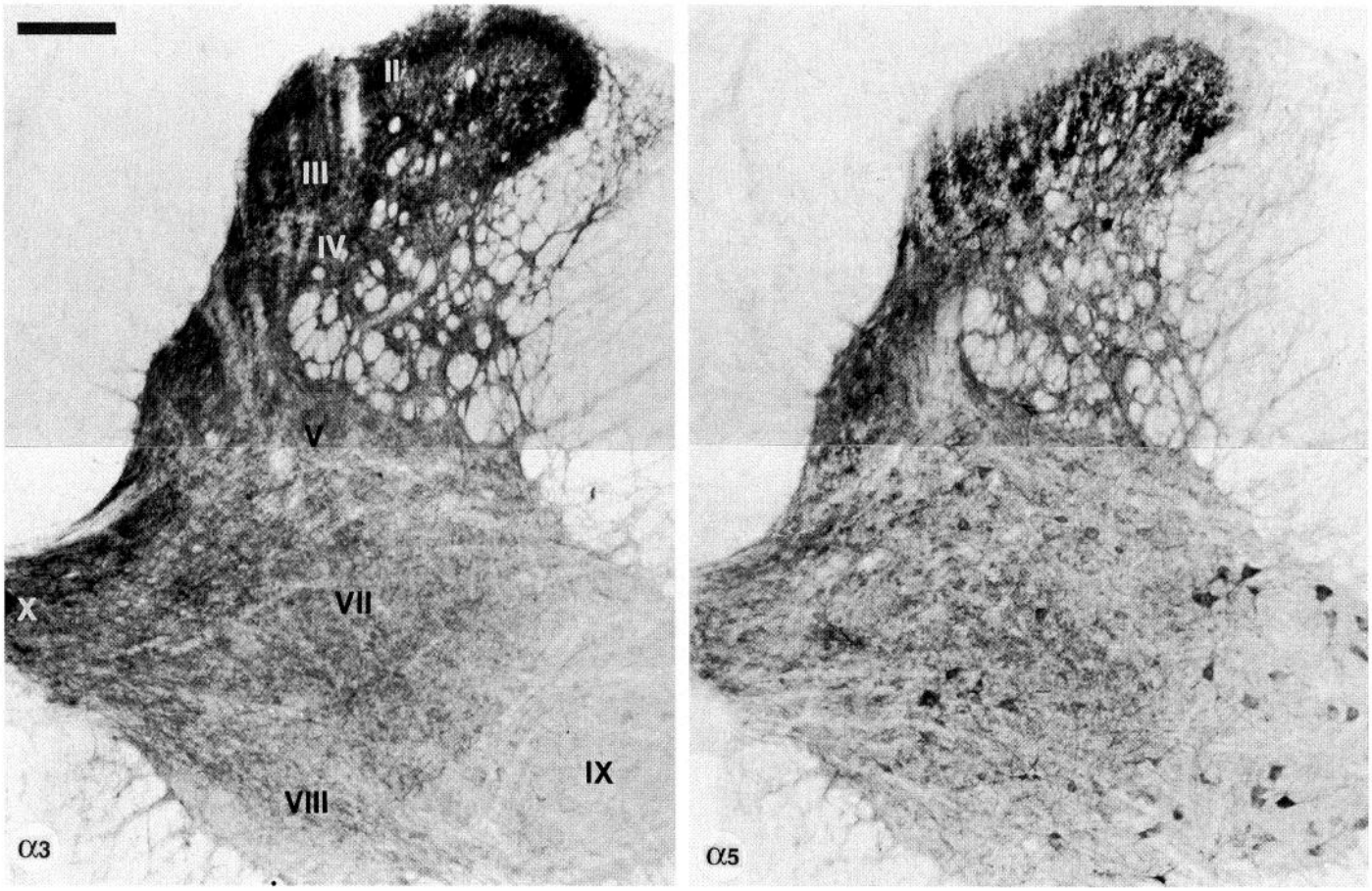

Figure 2. Distribution of the α3 and α5 subunits in the cervical spinal cord. The α3-subunit IR has the most widespread distribution among all α subunits, being present in all layers except layer IX. The α5-subunit IR is more discrete, being abundant in layers III–IV and moderate in layers V–IX. Like the α1-subunit IR, layers I–II appear devoid of α5-subunit IR. See Figures 1 and 3 for a comparison with the remaining subunits. Transverse sections were processed for immunoperoxidase staining with subunit-specific antisera. Scale bar, 200 μm.

puncta on the soma and dendrites (Fig. 5). For all the subunits, distinct aggregates of IR could be observed at high magnification on the cell surface, mostly on somata and proximal dendrites. These morphological features were markedly improved by the microwave pretreatment of the tissue (see Materials and Methods). Without the pretreatment, the background staining largely precluded the visualization of these aggregates. Variable amounts of intracellular staining also were observed, notably in large cells in laminae VII and VIII. The cells immunoreactive for the α1 subunit were very conspicuous, and their dendrites could often be followed over considerable distances in the transverse plane, extending into the lateral and ventral funiculi (Fig. 1). Based on their distribution and morphology, many of these cells are likely to represent spinoreticular and spinothalamic neurons, along with the large immunoreactive cells of laminae IV and V. These cells apparently also were stained for the β2,3 and γ2 subunits. In addition, the latter subunits were detected in numerous other cells, of variable size and morphology (Fig. 5*B*,*D*). Likewise, the α3-subunit IR labeled numerous neurons differing in shape and dendritic morphology. The α3 subunit was by far the most abundant α subunit variant in laminae VI–VIII, although it was not detected in all neurons (Fig. 5*A*).

Motoneurons (lamina IX) were intensely immunoreactive for the α2 and γ2 subunits. The α5-subunit IR also was seen in a subset of these cells, whereas the α1, α3, and β2,3 subunits could not be detected (Fig. 6). Typically, motoneurons exhibited a pronounced intracellular staining, which extended into proximal dendrites. In addition, the cell surface was apparently covered with a high density of aggregates of IR, which revealed the dendritic tree over considerable distances (Fig. 6*A*,*B*,*D*). In transverse sections, numerous dendritic profiles could be observed, forming rings of IR throughout lamina IX. The lack of α1-, α3-, and β2,3-subunit staining in motoneurons was particularly striking, because lamina IX was surrounded by neurons intensely immunoreactive for these subunits. A few fusiform neurons, putatively identified as Renshaw cells, were stained for the α1 and β2,3 subunits in lamina IX. Preganglionic motoneurons in the intermediolateral nucleus exhibited the same subunit repertoire as somatic motoneurons in lamina IX (data not shown).

In lamina X around the central canal, staining for all six subunits was observed, being most prominent for the α1, α3, β2,3, and γ2 subunits (Figs. 1–3).

The variable amounts of intracellular staining seen notably with the α3-, α5-, and γ2-subunit antisera are likely to be specific because they are seen only in immunopositive neurons (see Fig. 5*A* for the α3 subunit, for example). It is not established, however, whether they correspond to receptor proteins undergoing synthesis or degradation, or whether they represent a receptor reserve available for a rapid turnover.

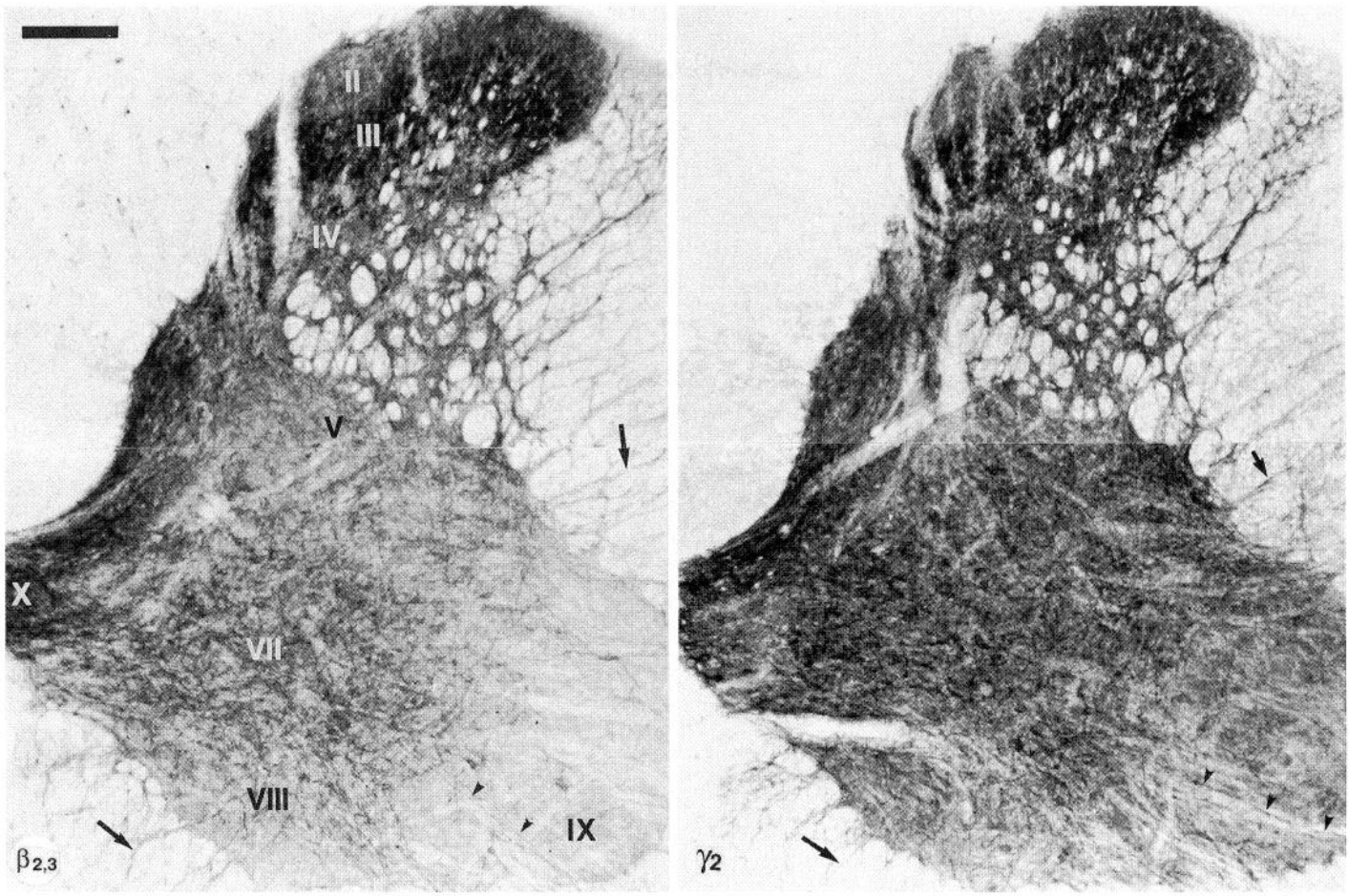

Figure 3. Ubiquitous distribution of the β2,3 and γ2 subunits in the cervical spinal cord. Except layer IX, devoid of β2,3-subunit IR, all spinal cord layers exhibit a moderate-to-intense staining for these subunits. Like the α1 subunit, the β2,3 and γ2 subunits also label neurons with dendrites oriented in the transverse plane (*arrowheads*) of the section, as well as dendrites penetrating into the white matter (*arrows*). See Figures 1 and 2 for a comparison with the remaining subunits. Transverse sections were processed for immunoperoxidase staining with subunit-specific antisera. Scale bar, 200 μm.

Colocalization of $GABA_A$-receptor subunits in individual neurons

The differential distribution of $GABA_A$-receptor subunits revealed by immunoperoxidase staining suggested that multiple receptor subtypes exist in the spinal cord. Insights into the subunit composition of these receptor subtypes were gained by double-immunofluorescence staining experiments, using the following nine combinations: α1/α2, α1/α3, α1/α5, α1/β2,3, α1/γ2, α2/β2,3, α3/β2,3, α5/β2,3, and γ2/β2,3. These combinations reflect the availability of antibodies raised in different species. The analysis had to be restricted, however, to those layers in which individual neurons could be distinguished unambiguously. Thus, the diffuse staining in laminae I, II, and X precluded a systematic evaluation of subunit colocalization in these layers.

The widespread expression of the β2,3 and γ2 subunits suggested that most receptor complexes comprise at least the triplet α/β/γ that forms the basis of functional receptors. This could be confirmed by double-immunofluorescence staining, because nearly 100% colocalization on the cellular level was observed between these two subunits and between the β2,3 subunits and the various α-subunit variants, as shown for the α1 subunit in Figure 7*A*. The most noticeable exception, as noted above, was observed in motoneurons, which apparently lacked the β2,3-subunit IR. In addition, it could be shown that all neurons expressing the α1-subunit IR were also positive for the γ2-subunit IR (Fig. 7*B*). The latter stained numerous additional cells, as expected from the contribution of the γ2 subunit to multiple receptor subtypes. The colocalization of the γ2-subunit IR with other α subunit variants could not be tested because these antisera were all raised in guinea pigs.

Further, the partially overlapping distribution of the α subunits suggested that certain neurons might express more than one α-subunit variant. However, only the α1-subunit antiserum (raised in rabbit) could be tested against the other α-subunit variants (raised in guinea pigs) (Figs. 8, 9). A semiquantitative evaluation of the extent of colocalization of the α-subunit variants is presented in Table 1. The most extensive colocalization of two α-subunit variants was observed between the α1 and α5 subunits (Fig. 8*A*). In lamina III, for instance, ~60% of the α1-subunit immunoreactive neurons also contained the α5 subunit. Likewise, the ratio reached 30% in laminae VII–VIII. The majority of α5-subunit-positive neurons nevertheless lacked the α1 subunit (Table 1), indicating that the overall number of neurons expressing the α5 subunit exceeds that of neurons expressing the α1 subunit. In stark contrast, the α1 and α3 subunits were never colocalized in the same neurons (Fig. 8*B*), although these two subunits showed considerable overlap in their regional distribution. This was particularly striking in laminae VII–VIII, where

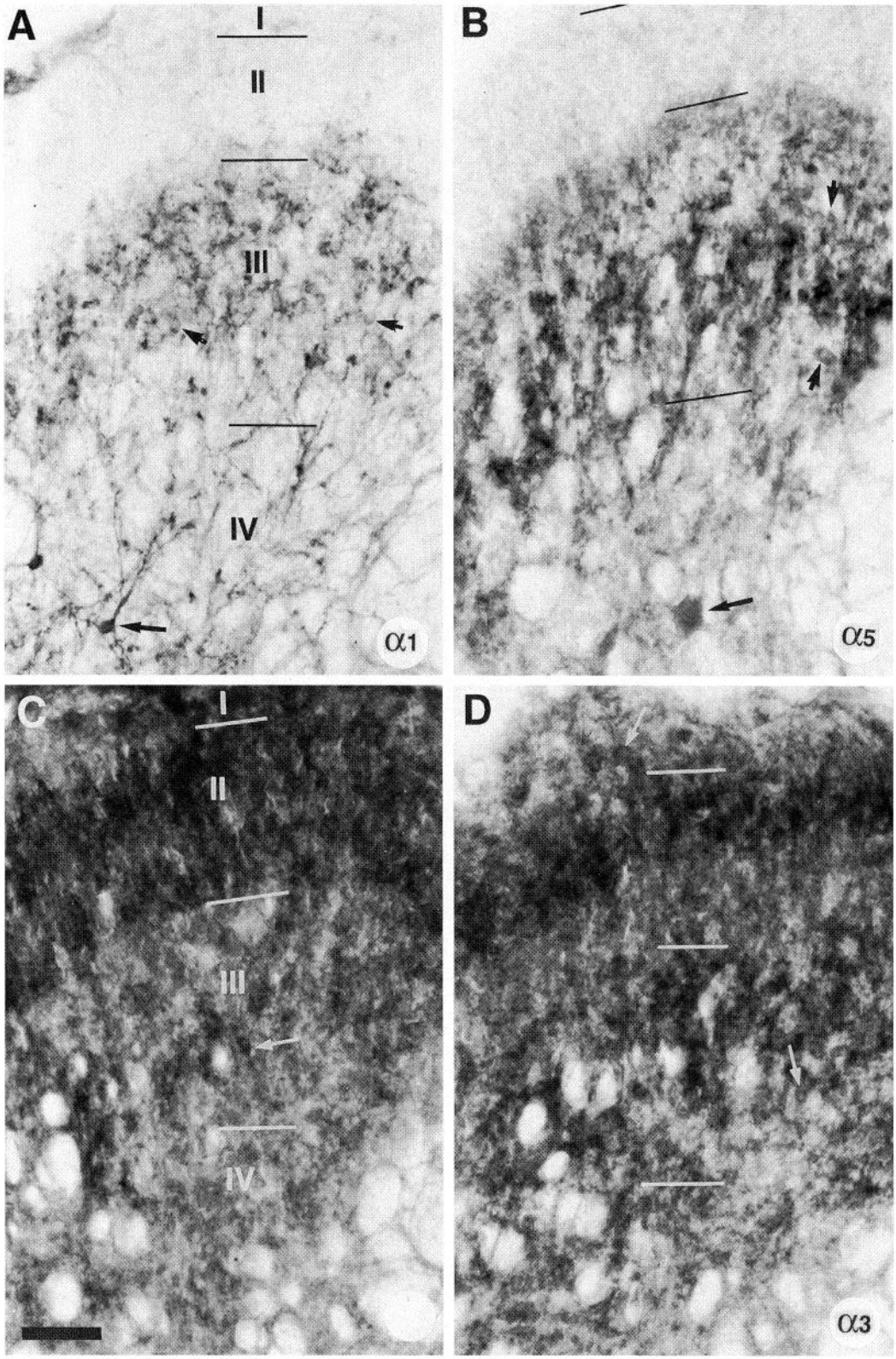

Figure 4. Differential distribution of the four α-subunit variants in the superficial layers of the dorsal horn. *A*, The α1-subunit IR is characterized by the selective labeling of small neurons in layer III (*short arrows*), located mostly in the superficial half of the layer. *B*, The α5-subunit IR is more extensive than the α1-subunit IR in layer III, notably in the deeper half, labeling both small and medium-sized neurons (*short arrows*). Both subunits label isolated neurons in layer IV (*long arrows*). *C* and *D*, The α2- and α3-subunit IR are characterized by a diffuse staining of layers I–III, which differs in intensity between the two subunits. The α2-subunit IR is uniform across layer II and only moderate in layer III, where a few neurons can be recognized (*arrow*), whereas the α3-subunit IR is most intense in the outer part of layer II and in layer III, where it labels numerous small neurons (*arrow*). Both subunits also are present in layer I, where individual positive cells can be seen (*arrow*). Scale bar, 50 μm.

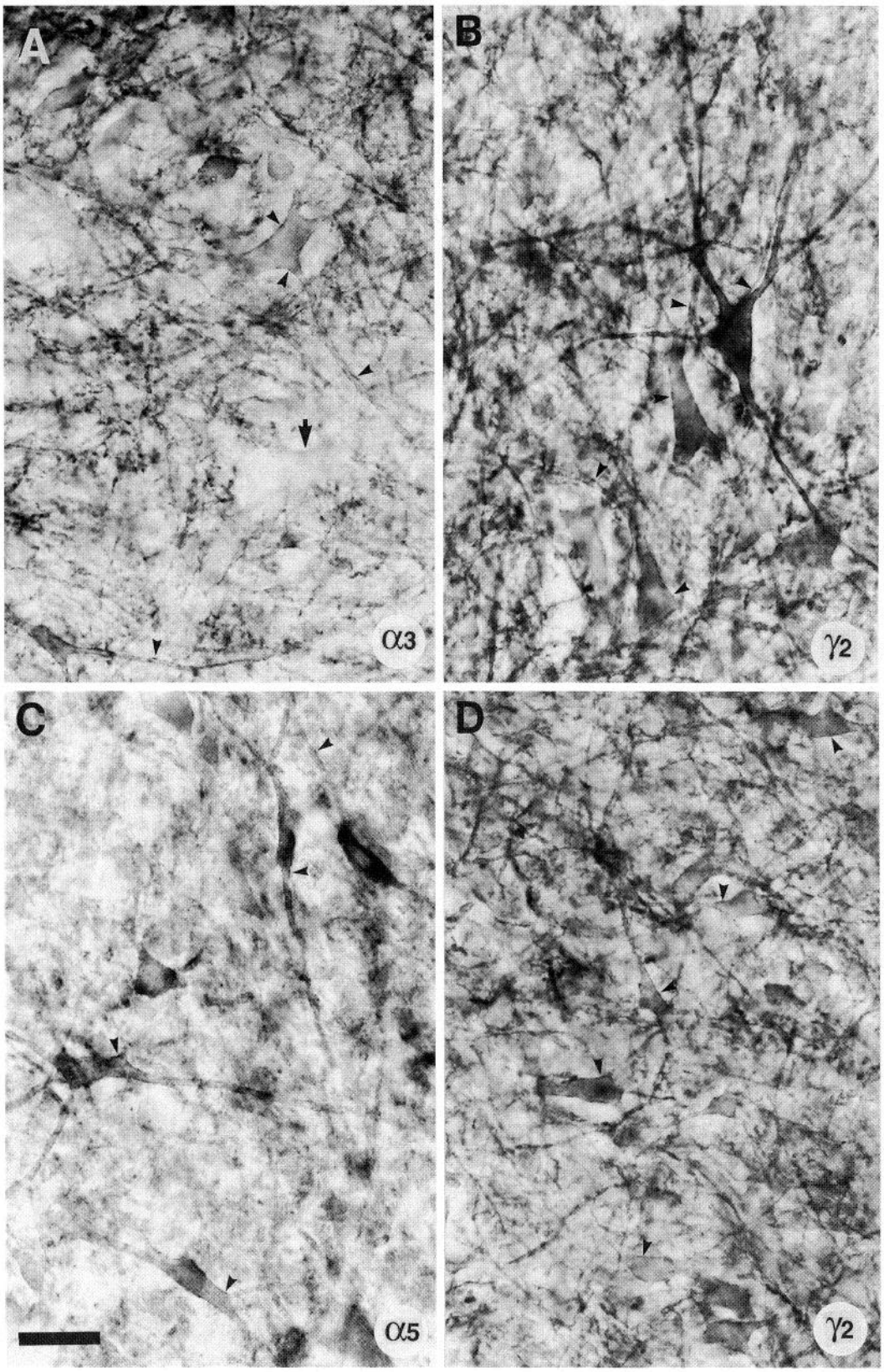

Figure 5. Cellular distribution of subunits in the ventral horn. *A* and *B*, Subunits α3 and γ2 in laminae VII. The α3-subunit antiserum moderately labels a subpopulation of neurons, producing a distinct, inhomogeneous staining of the surface of somata and dendrites (*arrowheads*). The *arrow* points to an unstained, large cell. The γ2-subunit IR is present on more neurons, and also forms aggregates over the soma and dendrites (*arrowheads*), which are strongly suggestive of postsynaptic aggregates. *C* and *D*, Subunits α5 and γ2 in lamina VIII. The α5-subunit IR is restricted to a few fusiform or multipolar large neurons, on which distinct aggregates are visible (*arrowheads*), whereas the γ2-subunit IR labels in addition numerous small neurons, all with an intense perisomatic labeling (*arrowheads*). Scale bar, 25 μm.

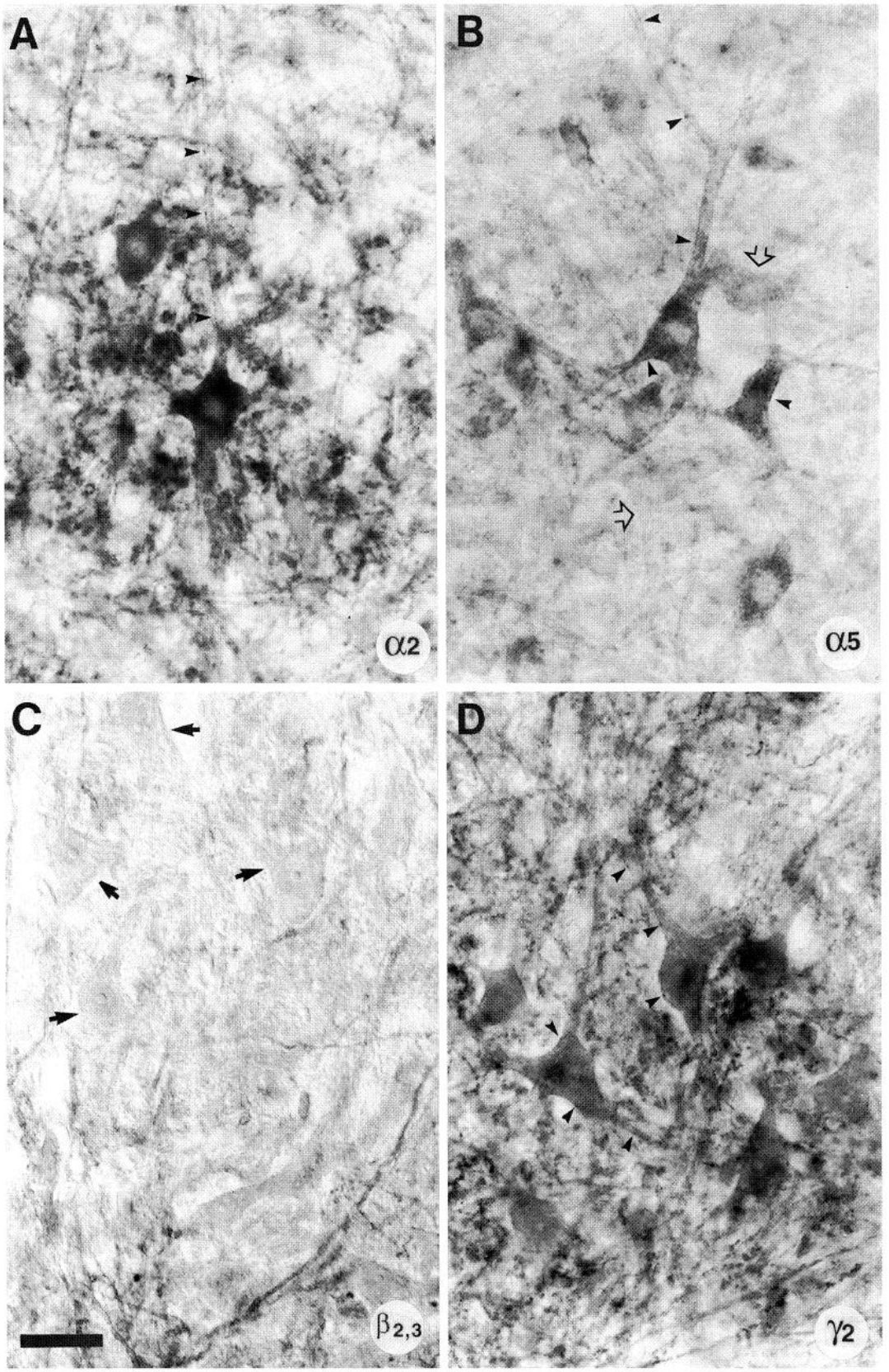

Figure 6. Cellular distribution of subunits in motoneurons. The α2- (*A*), α5- (*B*), and γ2-subunit (*D*) antisera label numerous presumptive motoneurons, producing a distinctive surface staining around the somata and dendrites (*arrowheads*). By contrast, the β2,3-subunit IR (*C*) was not detected in motoneurons (*arrows*). This panel was photographed with differential interference contrast to reveal the unstained cells. Whereas the α2- and γ2-subunit staining was seen in most motoneurons, the α5-subunit IR was prominent in only a subset of these cells and was weak (*open arrows*) or absent in others. Scale bar, 25 μm.

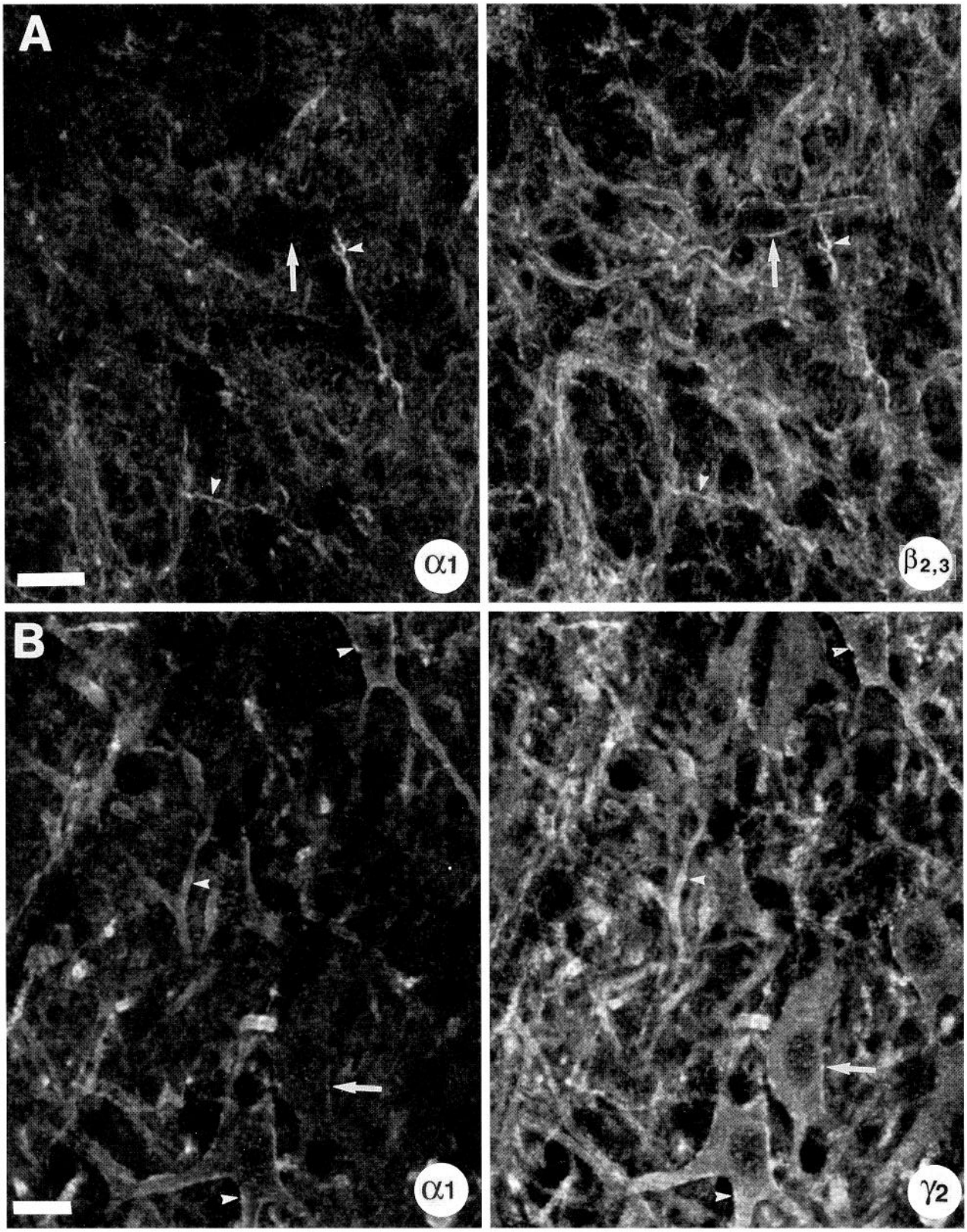

Figure 7. Video images from confocal microscopy display the partial colocalization of the α1 subunit with the β2,3 subunits (*A*) and the γ2 subunit (*B*) in sections processed for double-immunofluorescence staining. Each row depicts a pair of micrographs from the same section visualized with different filter combinations; in these micrographs, intense staining appears *white*. *A*, In layers IV–V, the β2,3-subunit IR is more abundant than the α1-subunit IR. The β2,3-subunit IR therefore labels neurons devoid of α1-subunit IR (*arrows*), whereas all dendrites stained for the α1 subunit are also positive for the β2,3 subunits (*arrowheads*). *B*, A similar pattern is evident in lamina VIII for the α1/γ2 combination, where the γ2-subunit IR is more widespread than the α1-subunit IR (*arrows*); some double-labeled neurons and dendrites are indicated by *arrowheads*. Scale bars: *A*, 20 μm; *B*, 10 μm.

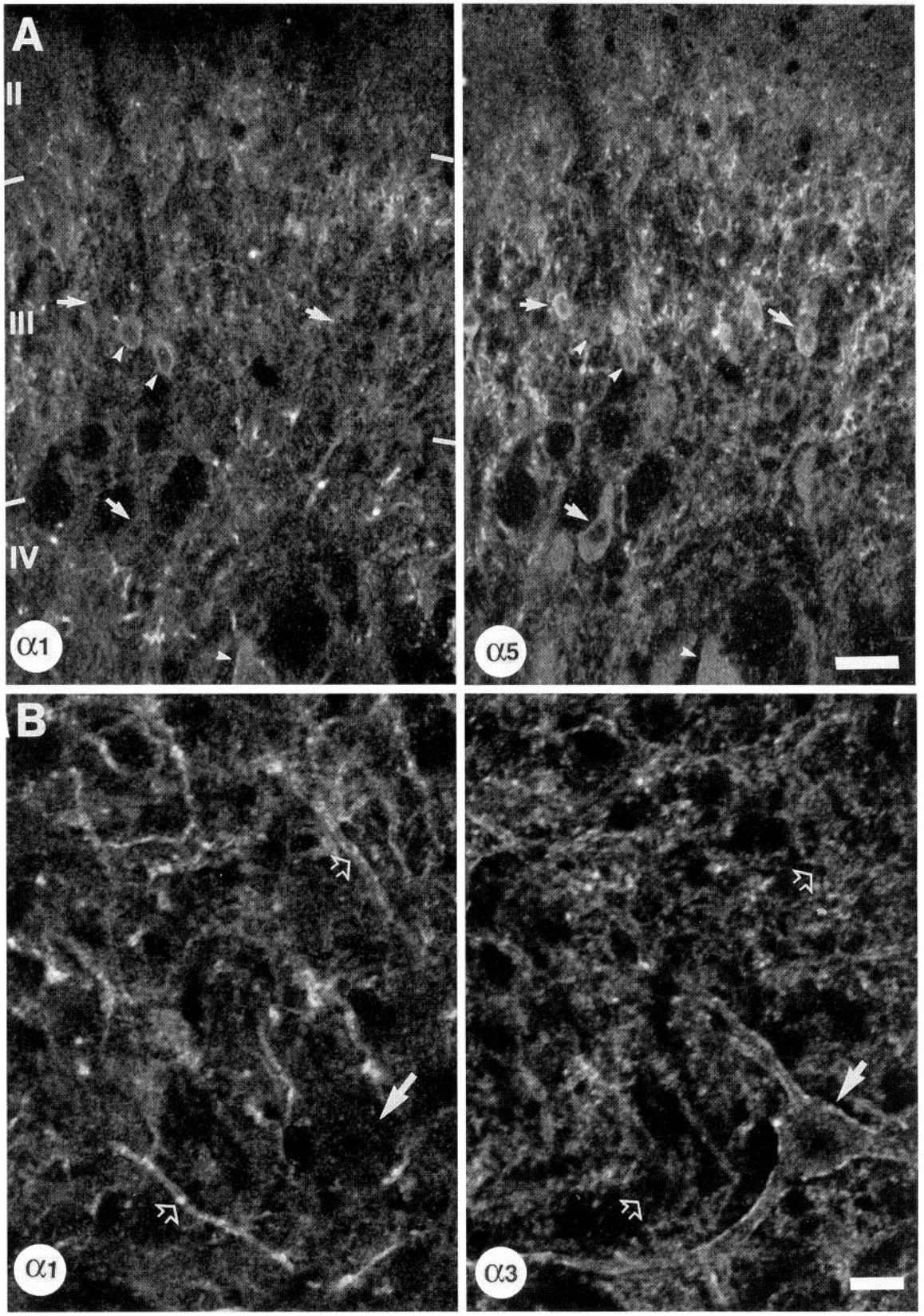

Figure 8. *A*, Partial colocalization of the α1- and α5-subunit IR in the superficial layers of the dorsal horn. Most α1-subunit-positive neurons are double-labeled for the α5 subunit (*arrowheads*). In addition, several cells appear stained for the α5 subunit only (*arrows*). *B*, Lack of colocalization of the α1- and α3-subunit IR in lamina VIII, where these subunits label different neuron populations. The *arrows* point to a neuron intensely immunoreactive for the α3 subunit (notice the numerous aggregates on the soma and dendrites) devoid of α1-subunit staining, whereas the *open arrows* depict dendrites stained for the α1 subunit only. Double-immunofluorescence staining visualized by confocal laser microscopy. Scale bars: *A*, 20 μm; *B*, 10 μm.

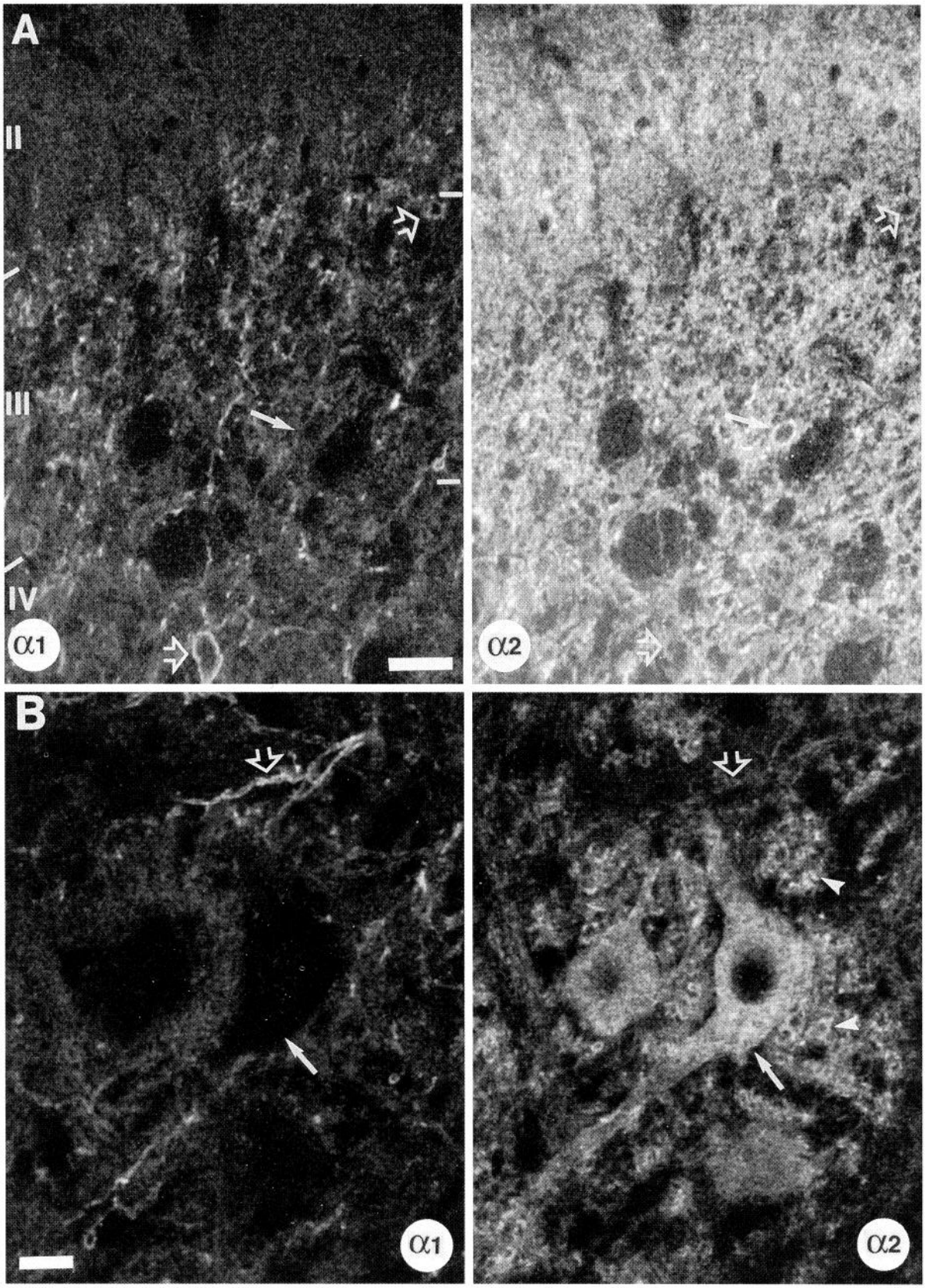

Figure 9. Lack of colocalization between the α1- and α2-subunit IR. *A*, In the superficial layers of the dorsal horn, the α2-subunit IR is very abundant, but is lacking in neurons positive for the α1 subunit (*open arrows*). Likewise, the somata positive for the α2 subunit are devoid of α1-subunit IR (*arrows*). *B*, In lamina IX, motoneurons are stained intensely for the α2 subunit, but lack the α1-subunit IR (*arrows*), which is present on only a few isolated dendrites (*open arrows*). *Arrowheads* in the α2-subunit section point to dendrites cut in cross-section. Double-immunofluorescence staining visualized by confocal laser microscopy. Scale bars: *A*, 20 μm; *B*, 10 μm.

neurons stained intensely for either subunit were abundant. In line with their largely nonoverlapping distribution, virtually no colocalization was observed between the α1 and α2 subunits (Table 1, Fig. 9), where the α1 subunit is most prevalent in the intermediate zone and the α2 subunit is most prevalent in laminae II–III and IX. Although the colocalization between the α2, α3, and α5 subunits could not be assessed directly, these three subunits labeled to a large extent morphologically distinct neurons, suggesting that they are probably not frequently coexpressed, except for the α2 and α5 subunits in a subset of motoneurons.

At least seven distinct subunit combinations, possibly representing seven distinct receptor subtypes, could be inferred from the colocalization patterns identified with the nine pairs of subunits tested. Although triple or quadruple subunit combinations could not be visualized directly, they are likely to occur based on the extent of colocalization of the respective subunit pairs. Thus, our results suggest that at least seven $GABA_A$-receptor subtypes exist in the spinal cord, characterized by the following subunit combinations: α3/β2,3/γ2; α2/β2,3/γ2; α1/β2,3/γ2; α5/β2,3/γ2; α1/α5/β2,3/γ2; α2/γ2; α2/α5/γ2. Each combination is expressed by a particular set of spinal cord neurons, distributed differentially in layers III–IX. Therefore, the structural heterogeneity of $GABA_A$ receptors correlates with the functional specialization of neurons in different spinal cord laminae.

DISCUSSION

The present results demonstrate an extensive heterogeneity in the subunit composition of $GABA_A$ receptors in the spinal cord. The unique, lamina-specific expression pattern of each subunit indicates that different $GABA_A$-receptor subtypes may be expressed in functionally distinct compartments of the spinal cord. This study extends previous findings in rat brain (Fritschy et al., 1992; Fritschy and Mohler, 1995) and retina (Greferath et al., 1995) and demonstrates that the major $GABA_A$-receptor subtypes detected in the brain are also present in the spinal cord.

Pattern of $GABA_A$-receptor subunit expression

In agreement with *in situ* hybridization studies (Persohn et al., 1991, 1992; Wisden et al., 1991; Ma et al., 1993), a predominant expression of the α3-, β2,3-, and γ2-subunit IR was detected in the spinal cord, along with the α2 subunit in motoneurons. In addition, however, a substantial α1- and α5-subunit IR was found, notably in lamina III and in the ventral horn, which is more intense than expected from the analysis of their mRNA levels (Ma et al., 1993). This discrepancy might reflect the fact that both subunits label neurons with an extensive dendritic tree, as seen for example in the α1 subunit in the ventral horn. Thus, the widespread immunohistochemical staining apparent at low magnification originates from a relatively small number of neurons that might not be very evident in autoradiograms.

The major discrepancy with *in situ* hybridization studies was observed, however, in laminae II–III, where the intense and diffuse α2-subunit IR contrasts with the lack of α2-subunit mRNA expression (Persohn et al., 1991, 1992; Wisden et al., 1991). This observation suggests that $GABA_A$ receptors containing the α2 subunit in the superficial dorsal horn are of extrinsic origin, being localized presynaptically on primary afferent terminals. Together with the α2 subunit, these receptors may contain the α3, β2,3, and γ2 subunits, which also exhibit a diffuse IR in the superficial dorsal horn. The expression of $GABA_A$ receptors in primary afferent terminals is in line with the strong mRNA expression of these subunits in dorsal root ganglion neurons (Persohn et al., 1991, 1992; Furuyama et al., 1992) and with functional studies demonstrating bicuculline-, picrotoxin-, and benzodiazepine-sensitive GABA-induced primary afferent depolarization in the spinal cord (Eccles et al., 1963; Barker and Nicoll, 1972; Polc, 1982; Gmelin and Zimmermann, 1983; Quevedo et al., 1992).

Ligand-binding and autoradiography studies revealed a high density of benzodiazepine (BZ) type II receptors in the spinal cord, notably in the superficial layers of the dorsal horn (Young and Kuhar, 1980; Villiger, 1984; Faull and Villiger, 1986; Santi et al., 1988). Because a BZ type II profile corresponds to $GABA_A$ receptors containing the subunits α2 or α3 (Pritchett et al., 1989; Benke et al., 1991; McKernan et al., 1991; Zezula and Sieghart, 1991; Marksitzer et al., 1993), these observations are in line with the intense staining for these subunits reported here in layers I–IV, and suggest that presynaptic receptors have this pharmacological profile. In addition, receptors insensitive to zolpidem, which are believed to contain the α5 subunit (Pritchett and Seeburg, 1990; Puia et al., 1991; Mertens et al., 1993), have been detected in the spinal cord (Ruano et al., 1992; Benavides et al., 1993; Mertens et al., 1993). Our results suggest that these receptors correspond to several subtypes, because the α5 subunit is associated in part with the α1 subunit (Table 1). An extensive pharmacological heterogeneity among $GABA_A$-receptor subtypes in the spinal cord therefore is to be expected.

Identification of $GABA_A$-receptor subtypes

To identify $GABA_A$-receptor subtypes, the colocalization of subunits within the soma and dendrites of individual neurons was considered to be indicative for the subunit composition of the respective receptors. Although the coexistence of subunits does not prove their coassembly into a single receptor subtype, we could not observe a differential distribution of two subunits on the subcellular level (e.g., in different aggregates). Thus, this analysis provides insight into the subunit repertoire of the major $GABA_A$-receptor subtypes expressed by spinal cord neurons, although we cannot rule out the possibility that certain neurons might express more than a single receptor subtype.

The vast majority of $GABA_A$-receptor subtypes in the spinal cord comprises at least the subunits α, β, and γ2, with the possible exception of motoneurons, which are apparently devoid of β2,3-subunit IR. The failure to detect β2,3-subunit staining in lamina IX was unexpected, because motoneurons express a prominent β3-subunit mRNA. However, a similar discrepancy has been observed also in cranial nerve motor nuclei (Fritschy and Mohler, 1995). It is not yet clear whether the β3-subunit protein is not translated in motoneurons or whether it is not detectable with bd-17 in these cells. Seven distinct subunit combinations could be inferred from the pattern of colocalization analyzed here, although triple and quadruple combinations could not be observed directly. For instance, the combination α3/β2,3/γ2 is expected to be present throughout the spinal cord, except in lamina IX, in view of the extensive coexistence of the α3/β2,3 subunits and γ2/β2,3 subunits. In addition, the subunit combinations α1/β2,3/γ2, α5/β2,3/γ2, and α1/α5/β2,3/γ2 are likely candidates representing distinct receptor subtypes distributed differentially in laminae III–VIII and X. The size, morphology, and distribution of neurons positive for the α1 subunit in laminae IV–VII suggest that they represent spinothalamic and spinoreticular neurons. In view of the evidence that $GABA_A$ receptors containing the α1 subunit correspond to BZ type I receptors (McKernan et al., 1991; Zezula and Sieghart, 1991), projection neurons thus might be characterized by the selective expression of BZ type I receptors. Motoneu-

rons may express the atypical combinations $\alpha2/\gamma2$ and $\alpha2/\alpha5/\gamma2$, although the latter could not be assessed directly. The presence of the $\alpha2$ subunit in these receptors is expected to confer them a BZ type II pharmacological profile (McKernan et al., 1991; Zezula and Sieghart, 1991).

There are several differences in the colocalization of α-subunit variants between the spinal cord and the brain (Fritschy and Mohler, 1995). In particular, the $\alpha1$ and $\alpha3$ subunits were almost never colocalized in spinal cord neurons, in spite of their overlap in laminae III, VII, and VIII, whereas several neuron populations coexpress these two subunits in the brainstem and basal forebrain (Gao et al., 1993, 1995; Fritschy and Mohler, 1995). Likewise, the $\alpha1$ and $\alpha5$ subunits co-occur in certain spinal cord neurons, although they are almost never colocalized in the brain (Fritschy and Mohler, 1995).

Our results reveal a striking segregation of $GABA_A$-receptor subtypes in functionally different neuron populations of the spinal cord. This is particularly evident in the dorsal horn, where the $\alpha2$ and/or $\alpha3$ subunits may represent receptors on afferent terminals and on local-circuit neurons in laminae II–III, whereas in laminae IV–VIII, receptors on projection neurons are represented primarily by the $\alpha1$ subunit. The putative significance of this heterogeneity for the mechanisms of nociception will be discussed in the next section.

Significance of $GABA_A$-receptor heterogeneity for spinal pain processing

It is generally accepted that benzodiazepines do not exert specific analgesic actions (Rodgers and Randall, 1987; Rosland et al., 1987). However, such properties are difficult to assess behaviorally because of the superimposed sedative and myorelaxant effects of benzodiazepines administered systemically. Recently, intravenous injection of midazolam was shown to induce a selective depression of noxiously evoked activity in rat spinal cord neurons (Clavier et al., 1992; Sumida et al., 1995), suggesting that this BZ modulates the transmission of nociceptive stimuli at segmental levels. Further, subarachnoid infusion of midazolam has been reported to be clinically effective for controlling intractable neoplastic pain (Schoeffler et al., 1991).

The differential expression of $GABA_A$-receptor subtypes between the superficial layers of the dorsal horn and projection neurons may be of particular relevance within the framework of the "gate control" theory (Melzack and Wall, 1965; Wall, 1980), which postulates that pain perception is modulated in the substantia gelatinosa (lamina II), which functions as a gate controlling impulse transmission from primary afferents to projecting neurons. GABAergic neurotransmission, which controls presynaptically the activity of afferent fibers and postsynaptically the excitation of interneurons in the substantia gelatinosa and of projecting neurons in the intermediate dorsal horn, has been proposed to play an important role in this mechanism. By demonstrating the presence of distinct $GABA_A$-receptor subtypes in the substantia gelatinosa and in projecting neurons, our results suggest that GABAergic transmission could be modulated differentially at these two levels by specific pharmacological agents.

Alterations in GABAergic neurotransmission at spinal levels may be of particular relevance for deafferentation pain syndromes and hyperalgesia, which develop after chronic peripheral lesions and inflammation (Sluka et al., 1993, 1994; Sivilotti and Woolf, 1994; Woolf and Doubell, 1994). These syndromes, collectively referred to as neural lesion pain, can be explained tentatively by the "gate control" theory. Under normal conditions, activation of the gate control system by large-diameter afferent fibers prevents innocuous cutaneous stimuli to be perceived as painful. After a lesion, this inhibitory control is relieved and non-noxious stimuli, such as light touch or vibration, can induce severe pain. The development of neural lesion pain thus may involve a decreased inhibitory control by GABAergic neurons, leading to a hyperactivity of dorsal horn neurons projecting to higher centers (Woolf and Doubell, 1994). Experimental support for this theory was provided by intrathecal administration of bicuculline in rats, which produces a decrease in pain threshold (allodynia). Thus, in animals treated with bicuculline, slight innocuous tactile stimuli induce an aversive behavior that is only weakly depressed by opioid administration (Yaksh, 1989; Sivilotti and Woolf, 1994). Because opioids are generally not effective in relieving neural lesion pain, these effects mimic those observed clinically after deafferentation. Further, bicuculline enforced the hyperalgesia induced by chronic nerve compression, a model of neuropathic pain in rats (Yamamoto and Yaksh, 1993), and also was found to reduce the analgesic action of dorsal column stimulation, a procedure used for the treatment of chronic pain (Duggan and Foong, 1985). It therefore is conceivable that the pharmacological control of nociception and the treatment of neurogenic pain could be based, in the future, on $GABA_A$-receptor heterogeneity. When the subunit composition of native receptor subtypes in the substantia gelatinosa and in projecting cells is determined definitively, the functional characterization of these receptor subtypes should provide insights into the significance of receptor heterogeneity in sensory processing and pain mechanisms. Ultimately, these studies may lead to the development of subtype-specific receptor-ligands acting selectively at different levels of the spinal cord to produce effective analgesia without undesirable central side effects.

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