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Localization of Ca^{2+} Channel Subtypes on Rat Spinal Motor Neurons, Interneurons, and Nerve Terminals

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Ca^{2+} channels in distinct subcellular compartments of neurons mediate voltage-dependent Ca^{2+} influx, which integrates synaptic responses, regulates gene expression, and initiates synaptic transmission. Antibodies that specifically recognize the α_1 subunits of class A, B, C, D, and E Ca^{2+} channels have been used to investigate the localization of these voltage-gated ion channels on spinal motor neurons, interneurons, and nerve terminals of the adult rat. Class A P/Q-type Ca^{2+} channels were present mainly in a punctate pattern in nerve terminals located along the cell bodies and dendrites of motor neurons. Both smooth and punctate staining patterns were observed over the surface of the cell bodies and dendrites with antibodies to class B N-type Ca^{2+} channels, indicating the presence of these channels in the cell surface membrane and in nerve terminals. Class C and D L-type and class E R-type Ca^{2+} channels were distributed mainly over the cell soma and proximal dendrites. Class A P/Q-type Ca^{2+} channels were present predominantly in the presynaptic terminals of motor neurons at the neuromuscular junction. Occasional nerve terminals innervating skeletal muscles from the hindlimb were labeled with antibodies against class B N-type Ca^{2+} channels. Staining of the dorsal laminae of the rat spinal cord revealed a complementary distribution of class A and class B Ca^{2+} channels in nerve terminals in the deeper versus the superficial laminae. Many of the nerve terminals immunoreactive for class B N-type Ca^{2+} channels also contained substance P, an important neuropeptide in pain pathways, suggesting that N-type Ca^{2+} channels are predominant at synapses that carry nociceptive information into the spinal cord.

Key words: Ca^{2+} channels; spinal cord; motor neurons; interneurons; nerve terminals; substance P

Spinal motor neurons are the final integration point for electrical signals that initiate and control skeletal muscle contraction. Ca^{2+} channels play a critical role in this integration process. Several neuromuscular diseases result from dysfunction of the motor neurons. In at least two cases, Ca^{2+} channels are implicated in the disease process. Lambert-Eaton myasthenic syndrome is caused by circulating antibodies against presynaptic Ca^{2+} channels (Engel, 1991; Sher et al., 1993). These antibodies reduce the level of presynaptic Ca^{2+} current and the efficiency of neurotransmitter release (Lang et al., 1983; Kim, 1985). Amyotrophic lateral sclerosis (ALS) is caused by progressive death of motor neurons (Appel and Stefani, 1991). One current hypothesis for the etiology of ALS implicates Ca^{2+} channels in motor neurons (Appel et al., 1991, 1993; Delbono et al., 1991, 1993; Smith et al., 1992; Uchitel et al., 1992; Morton et al., 1994; Rowland, 1994).

On the basis of pharmacological and physiological properties, at least six distinct types of voltage-gated Ca^{2+} channels have been identified and are designated L, N, P, Q, R, and T (Bean, 1989; Llinas et al., 1989; Hess, 1990; Zhang et al., 1993; Randall and Tsien, 1995). Multiple isoforms of the principal α_1 subunit of voltage-gated Ca^{2+} channels, designated class A–E, have been cloned from rat brain (Snutch et al., 1990; for review, see Snutch and Reiner, 1992; Soong et al., 1993; Zhang et al., 1993; Birnbaumer et al., 1994). The rat brain class C and D genes encode L-type Ca^{2+} channel α_1 subunits, which are ~75% identical in amino acid sequence with those of rabbit skeletal muscle Ca^{2+} channels (Hui et al., 1991; Snutch et al., 1991; Chin et al., 1992; Seino et al., 1992; Williams et al., 1992a; Tomlinson et al., 1993). Class C and D Ca^{2+} channels have high affinity for dihydropyridine Ca^{2+} channel antagonists and have been shown to be localized predominantly in the soma and proximal dendrites of neurons throughout the brain (Hell et al., 1993) where they are important for regulation of gene expression (Murphy et al., 1991; Bading et al., 1993; Bito et al., 1996; Deisseroth et al., 1996). The α_{1B} subunit is localized predominantly in dendritic shafts and presynaptic terminals (Westenbroek et al., 1992) and forms an N-type, high-voltage-activated Ca^{2+} channel having high affinity for ω-conotoxin GVIA (Dubel et al., 1992; Williams et al., 1992b; Fujita et al., 1993). Class A channels containing α_{1A} subunits (Mori et al., 1991; Starr et al., 1991; Stea et al., 1994) are blocked by ω-agatoxin IVA and ω-conotoxin MVIIC. Their functional and pharmacological properties closely resemble Q-type Ca^{2+} channels, which have been described in cerebellar granule cells (Zhang et al., 1993; Stea et al., 1994; Randall and Tsien, 1995), and P-type Ca^{2+} channels in cerebellar Purkinje cells and other neurons (Llinas et al., 1989; Mintz et al., 1992; Stea et al., 1994; Westenbroek et al., 1995). The class A channels are localized predominantly in presynaptic terminals and dendritic shafts in brain neurons (Westenbroek et al., 1995). Class E Ca^{2+} channel subunits (Soong et al., 1993) are localized mainly in cell bodies and less frequently in dendrites of neurons in the CNS (Yokoyama et al., 1995) and have some features of a low-voltage-activated R-type Ca^{2+} channel (Soong et al., 1993; Zhang et al., 1993).

Despite the important role of Ca^{2+} channels in spinal motor neurons and interneurons, detailed information on the subcellu-

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lar distribution of voltage-gated Ca^{2+} channels in their dendrites, cell soma, and nerve terminals is lacking. Spinal motor neurons also provide an opportunity for analysis of the distribution of Ca^{2+} channels in the central cell body and dendrites compared with the peripheral nerve terminals of a single class of neurons. In these experiments, we have used specific antibodies to define the distribution of five Ca^{2+} channel subtypes on spinal motor neurons and interneurons and their nerve terminals.

MATERIALS AND METHODS

Antibodies. Antibodies that specifically recognize the α_1 subunits of class A (anti-CNA1, anti-CNA5, and anti-CNA6 antibodies), class B (anti-CNB2 antibodies), class C (anti-CNC1 antibodies), class D (anti-CND1 antibodies), and class E (anti-CNE2 antibodies) Ca^{2+} channels were used in this study. Their generation, purification, and characterization have been reported previously (Westenbroek et al., 1990, 1992, 1995; Hell et al., 1993; Yokoyama et al., 1995; Sakurai et al., 1996). The antibodies to synaptotagmin (1D12) and syntaxin (10H5) were generous gifts of Dr. Masami Takahashi (Mitsubishi-Kasei Life Sciences Institute, Tokyo, Japan). The antibody to substance P was obtained from Genosys (The Woodlands, TX). Avidin, biotin, biotinylated anti-rabbit IgG, biotinylated anti-mouse IgG, biotinylated anti-goat IgG, avidin D–fluorescein, Vectashield, and anti-mouse IgG tagged with fluorescein were purchased from Vector (Burlingame, CA).

Immunocytochemistry. Adult Sprague Dawley rats were anesthetized with Nembutal and perfused intracardially with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.36% lysine and 0.05% sodium *m*-periodate. The spinal cord, diaphragm, tibialis anterior muscle, soleus muscle, and gastrocnemius muscle were immediately removed and post-fixed for 2 hr. The tissue was then cryoprotected in 10% (w/v) sucrose for 12 hr and 30% (w/v) sucrose for 48 hr. Tissue sections (35 μm) were cut on a sliding microtome and placed in 0.1 M phosphate buffer.

Single-labeling studies. Tissue sections were rinsed in 0.1 M Tris buffer (TB), pH 7.4, for 20 min, in 0.1 M Tris buffered saline (TBS), pH 7.4, for 20 min., blocked using 2% avidin in TBS for 30 min, rinsed in TBS for 30 min, blocked using 2% biotin for 30 min, and finally rinsed in TBS for 30 min. The tissue sections were then incubated in affinity-purified anti-CNA1 (diluted 1:15), anti-CNA5 (diluted 1:25), anti-CNA6 (diluted 1:25), anti-CNB2 (diluted 1:15), anti-CNC1 (diluted 1:15), anti-CND1 (diluted 1:15), anti-CNE2 (diluted 1:15), or anti-synaptotagmin (diluted 1:200) for 36 hr at 4°C. All antibodies were diluted in a solution containing 0.075% Triton X-100 and 1% NGS in 0.1 M TBS. The tissue sections were rinsed in TBS for 60 min and incubated in biotinylated goat anti-rabbit IgG (for sections incubated in Ca^{2+} channel antibodies) or biotinylated goat anti-mouse IgG (for sections incubated with anti-synaptotagmin) diluted 1:300 for 1 hr at 37°C. The tissue sections were rinsed with TBS for 60 min and incubated in avidin D–fluorescein diluted 1:300 for 1 hr at 37°C. The sections were rinsed in TBS for 10 min, rinsed in TB for 20 min, and then mounted on gelatin-coated coverslips, coverslipped with Vectashield, sealed with nail polish, and viewed with a Bio-Rad MRC 600 microscope located in the W. M. Keck Imaging Facility at the University of Washington.

Double-labeling studies. Sections were fixed, sliced, rinsed, and blocked as described above. Muscle sections were then incubated in anti-CNA1 and anti-synaptotagmin or anti-CNB2 and anti-synaptotagmin at the same time for 36 hr at 4°C. Sections from the spinal cord were incubated in anti-CNA1 and anti-substance P (diluted 1:200), anti-CNB2 and anti-substance P, anti-CNA1 and anti-syntaxin (diluted 1:200), or anti-CNB2 and anti-syntaxin at the same time for 36 hr at 4°C. The tissue was rinsed in TBS for 1 hr and then incubated in biotinylated anti-rabbit IgG (diluted 1:300), which will recognize the Ca^{2+} channel antibodies, or in anti-mouse IgG-rhodamine (diluted 1:100), which will recognize the anti-synaptotagmin, anti-syntaxin, and anti-substance P antibodies, for 1 hr at 37°C. Tissue was rinsed in TBS for 1 hr and incubated in avidin D–fluorescein (diluted 1:300) for 1 hr. The sections were rinsed with TBS for 10 min, rinsed with TB for 20 min and then mounted, coverslipped, and viewed as described above.

Spinal cord sections double-labeled for class A and class B Ca^{2+} channels were fixed, sliced, rinsed, and blocked as described above. The sections were incubated in anti-CNA1 (made in rabbit; diluted 1:15) for 36 hr. The tissue was rinsed for 1 hr and incubated in biotinylated anti-rabbit IgG (diluted 1:300) for 1 hr at 37°C. The tissue was rinsed in TBS for 1 hr and incubated in avidin D–fluorescein (diluted 1:300) for 1 hr. The tissue was rinsed in TBS for 40 min, blocked using 2% avidin and 2% biotin as described above, rinsed in TBS for 30 min, and blocked in TBS containing 10% normal goat serum for 1 hr. The spinal cord sections were then incubated in anti-CNB1 (made in goat; diluted 1:15) for 36 hr at 4°C. The tissue was then rinsed in TBS for 1 hr, incubated in biotinylated anti-goat IgG (diluted 1:300) for 1 hr at 37°C, rinsed for 1 hr in TBS, and then incubated in avidin D–rhodamine (diluted 1:200) for 1 hr at 37°C. Finally, the tissue was rinsed in TBS for 10 min, then in TB for 20 min, mounted on gelatin-coated slides, and coverslipped using Vectashield. Control sections were incubated in normal rabbit serum, or the primary antibody was omitted. In both instances, no specific staining was observed.

RESULTS

The localization of the α_1 subunits of class A, B, C, D, and E Ca^{2+} channels in the surface membrane of the cell bodies, dendrites, and terminals of spinal motor neurons was investigated along the length of the spinal cord. Our results indicate that the distribution of these different types of the Ca^{2+} channels over the cell bodies and dendrites of motor neurons and interneurons does not vary substantially with the level of the spinal cord at which they are located. Thus, the following descriptions apply to motor neurons along the entire rostral-caudal extent of the spinal cord.

Distribution of class A–E Ca^{2+} channels on the soma and dendrites of spinal motor neurons

The antibodies used in these studies have been previously characterized with respect to specificity and immunoreactivity and shown to specifically label the class A–E α_1 subunits (Westenbroek et al., 1990, 1992, 1995; Hell et al., 1993; Yokoyama et al., 1995). Staining for the α_1 subunit of class A Ca^{2+} channels using anti-CNA1 antibodies was found throughout the ventral horn (Fig. 1*A*), in regions surrounding the spinal motor neurons. There is dense punctate staining along the surface of motor neuron cell bodies and dendrites (Fig. 1*B,C*). This punctate pattern of staining with the anti-CNA1 antibody has been shown previously in various brain regions to be associated with nerve terminals (Westenbroek et al., 1995) (see Fig. 6*C*). A similar pattern of staining was observed along the surface of interneurons in all laminae of the spinal cord. Dense staining of terminals was also observed in the surrounding neuropil (Fig. 1*B,C*). These results are consistent with the conclusion that α_{1A} subunits are primarily localized in nerve terminals forming synapses on motor neurons and interneurons.

With anti-CNB2 antibodies, the α_1 subunits of class B Ca^{2+} channels were localized to the cell bodies and dendrites of neurons residing in the ventral horn of the spinal cord (Fig. 1*D*). The staining along the surface of motor neurons is both smooth and punctate in appearance (Fig. 1*E,F*), consistent with the presence of α_{1B} in the cell surface of dendrites and somata as well as in nerve terminals forming synapses on them (see Fig. 6*D*). Of the nerve terminals in the neuropil surrounding the motor neurons, a lower density was stained with the class B antibodies than with the class A antibodies (Fig. 1, compare *B* and *C* with *E* and *F*).

The distributions of the α_1 subunits of class C, class D, and class E Ca^{2+} channels (Fig. 2*A–F*) were similar to their distributions in neurons in many brain regions (Hell et al., 1993; Yokoyama et al., 1995), with localization predominantly in the cell soma and proximal dendrites of the spinal motor neurons. Sections stained with anti-CNC1 antibodies to the α_1 subunit of class C L-type Ca^{2+} channels were immunoreactive throughout the ventral horn (Fig. 2*A*). A combination of smooth and punctate staining was observed over the cell soma and along the proximal dendrites of motor neurons and interneurons (Fig. 2*B*). This pattern of stain-

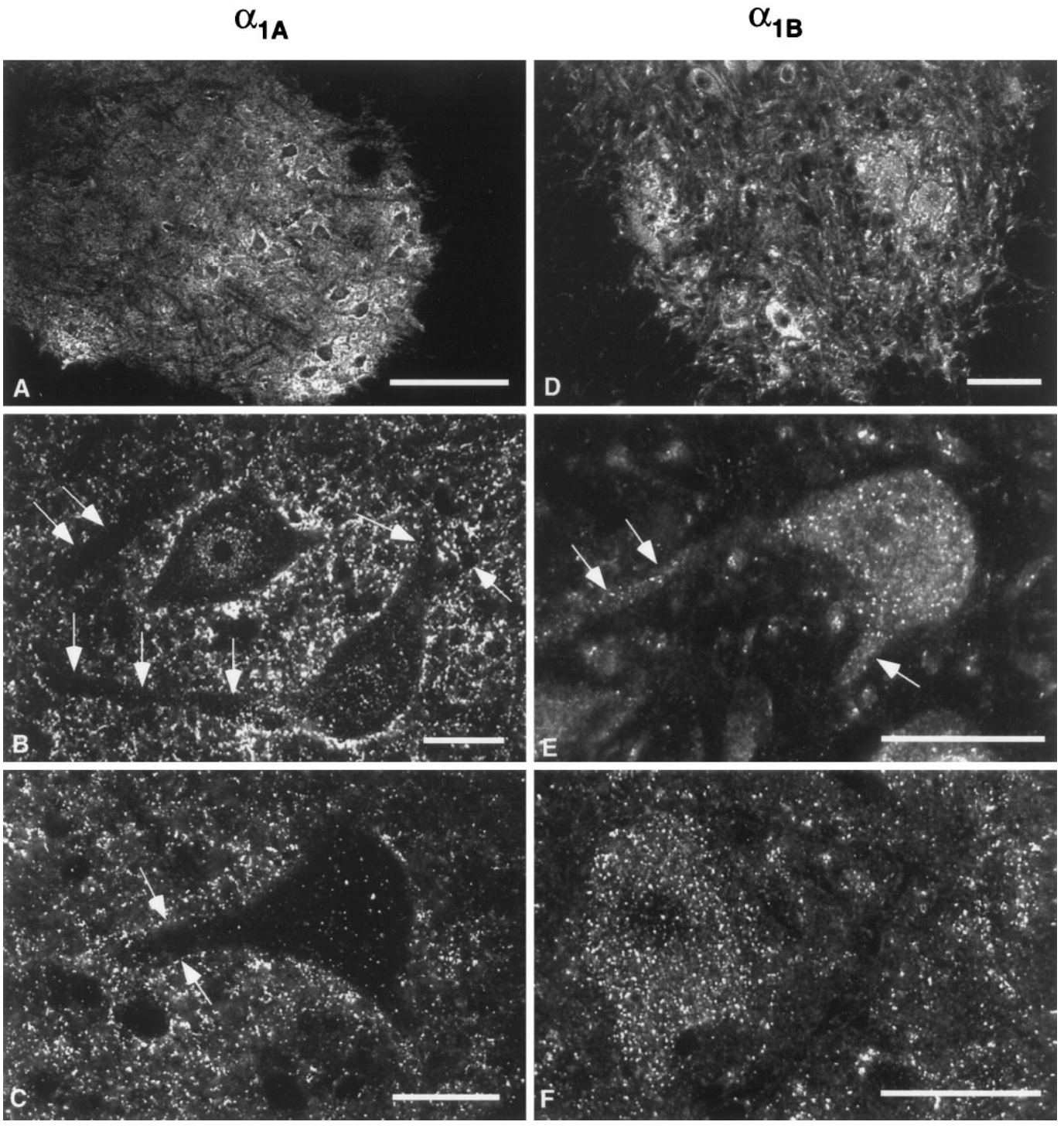

Figure 1. Distribution of class A and class B Ca^{2+} channels in the ventral horn. *A–C*, Tissue sections incubated with anti-CNA1 antibodies to the class A Ca^{2+} channels illustrating staining of terminals throughout the ventral horn and along the cell bodies and dendrites of motor neurons. *Arrows* point to dendritic regions. *D–F*, Tissue sections incubated with anti-CNB2 antibodies in the ventral horn of the spinal cord demonstrating both smooth and punctate staining along the cell soma and dendrites of motor neurons. *Arrows* point to regions of dendritic staining. Scale bars: *A*, 250 μm; *B, C, E, F*, 25 μm; *D*, 50 μm.

ing with anti-CNC1 has been shown previously to be localized in postsynaptic sites (Hell et al., 1996). Staining in the surrounding neuropil appeared to be along dendritic surfaces, similar to that observed along the dendrites of hippocampal CA3 pyramidal neurons (Hell et al., 1993). Tissue sections incubated with anti-CND1 antibodies to the class D L-type Ca^{2+} channels revealed a smooth distribution of immunoreactive α_{1D} subunits over the surface of the soma and the proximal dendrites similar to that observed in other brain regions (Hell et al., 1993). Ca^{2+} channels containing α_{1D} were present on the surface of neurons throughout the ventral horn (Fig. 2*C,D*). The dendritic immunoreactivity was relatively weak, and very little staining was observed in the

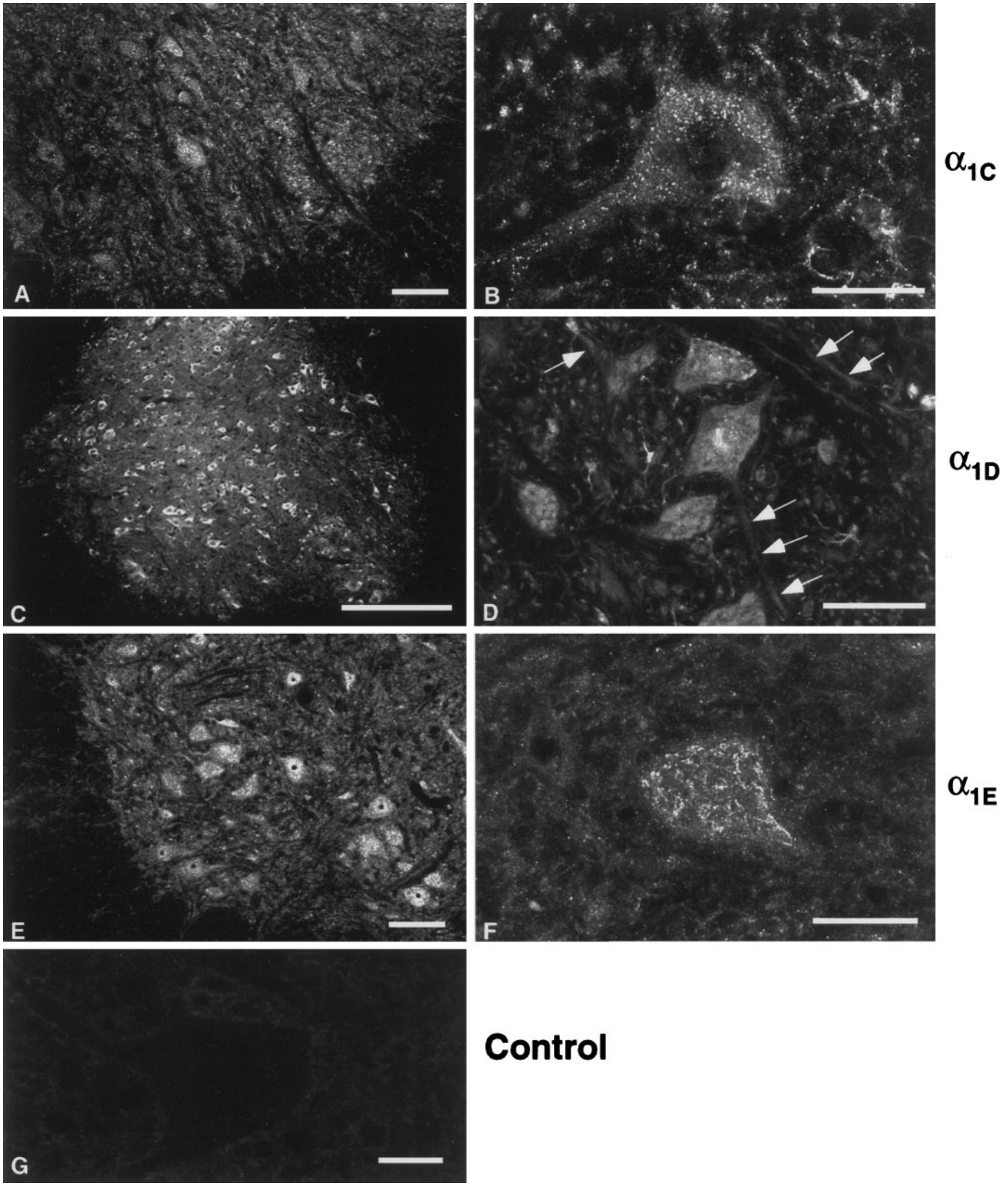

Figure 2. Localization of class *C–E* Ca^{2+} channels in the ventral horn. *A, B*, Tissue sections labeled with anti-CNC1 antibodies illustrating the punctate pattern of staining along the cell surface and the proximal dendrites. *C, D*, Tissue sections labeled with anti-CND1 antibodies demonstrating the presence of class D channels mainly on the cell body and proximal dendrites. *Arrows* indicate regions of dendritic staining. *E, F*, Sections stained with anti-CNE2 antibodies showing their presence mainly along the cell bodies of spinal motor neurons. *G*, Control section incubated with normal rabbit serum to illustrate the lack of specific staining. Scale bars: *A, D, E*, 50 μm; *B, F, G*, 25 μm; *C*, 250 μm.

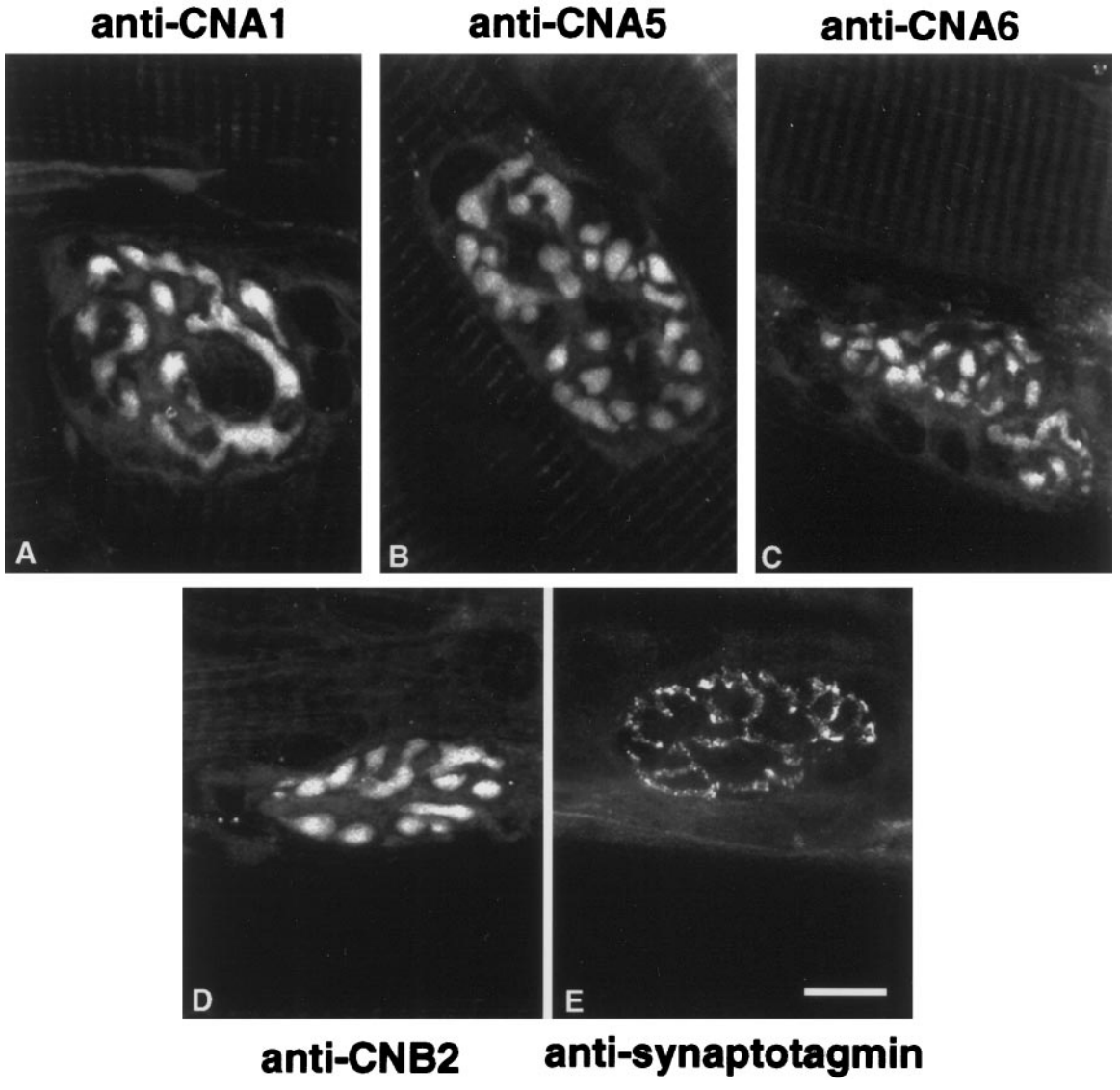

Figure 3. Ca^{2+} channels in nerve terminals. Diaphragm muscle stained with anti-CNA1 (*A*) or with anti-CNA5 (*B*). Tibialis anterior muscle stained with anti-CNA6 (*C*), anti-CNB2 (*D*), or anti-synaptotagmin (*E*) illustrating the presence of both class A and class B Ca^{2+} channels at the NMJ. Scale bar, 10 μm.

surrounding neuropil (Fig. 2*D*). Thus, α_{1D} is primarily localized in the cell bodies of spinal motor neurons and interneurons. Class E Ca^{2+} channel immunoreactivity was both smooth and clustered in extended arrays over the cell body of motor neurons, as visualized with anti-CNE2 Ca^{2+} channel antibodies (Fig. 2*E,F*). Relatively weak, punctate staining was observed in the surrounding neuropil with the anti-CNE2 antibodies (Fig. 2*F*). Control sections incubated with normal rabbit serum were not labeled (Fig. 2*G*). A similar lack of staining was observed when the primary antiserum was omitted.

Localization of class A–E Ca^{2+} channels in motor neuron terminals

Skeletal muscles are innervated by motor neurons whose cell bodies reside in the spinal cord or brain stem. Each motor neuron sends an axon to a single muscle where it then branches to innervate many muscle fibers. Our results indicate that class C, class D, and class E α_1 subunits are not present at adult rat neuromuscular junctions (NMJs) in the diaphragm, tibialis anterior, soleus, or gastrocnemius muscles in densities detectable by our anti-peptide antibodies (data not shown). Among the skeletal muscles we examined, the predominant α_1 subunit of Ca^{2+} channels observed at the neuromuscular junction was α_{1A} (Fig. 3*A*) as detected using the anti-CNA1 antibody, which recognizes both isoforms of α_{1A} (Sakurai et al., 1996). Using antibodies that distinguish between the rbA and BI isoforms of α_{1A} [anti-CNA5 and anti-CNA6 (Sakurai et al., 1996)], we observed that both the rbA (Fig. 3*B*) and BI (Fig. 3*C*) are present at the adult rat NMJ in approximately equal abundance. These channels were observed in NMJs of the diaphragm, tibialis anterior, soleus, and gastrocnemius muscles. In addition to the presence of class A Ca^{2+} channels at the NMJ, we also observed staining with anti-CNB2 antibodies for class B N-type channels (Fig. 3*D*). Terminals labeled with anti-CNB2 were in low abundance (2–5% of total labeled) compared with those stained with anti-CNA1. The terminals stained by anti-CNB2 were observed only in the tibialis anterior, gastrocnemius, and soleus muscles in this study,

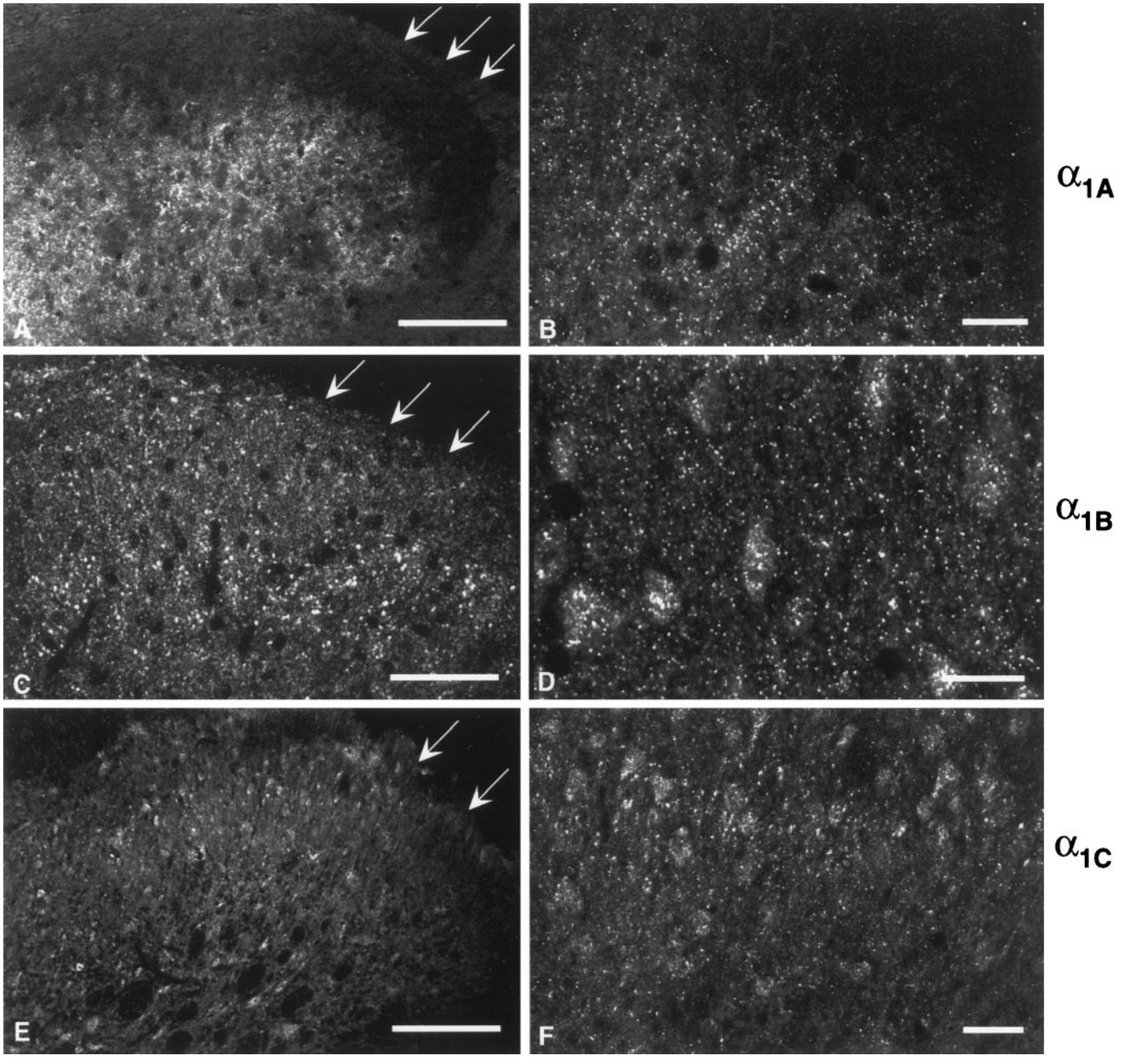

Figure 4. Localization of Ca^{2+} channels in the dorsal horn. *A, B*, Sections stained with anti-CNA1 illustrating the labeling of terminals in the superficial layers of the cord. *Arrows* point to the dorsal surface of the spinal cord. *C, D*, Tissue sections incubated with anti-CNB2 antibodies showing labeling of terminals and cell bodies in the superficial laminae of the spinal cord. *E, F*, Sections labeled with anti-CNC1 antibodies demonstrating punctate immunoreactivity associated with cell body in the dorsal horn. Scale bars: *A, C, E*, 100 μm; *B, D*, 10 μm; *F*, 50 μm.

and no staining with anti-CNB2 antibodies was observed at the NMJs located in the diaphragm. To confirm the presence of class A and class B channels at the NMJ, tissue sections were also stained with anti-synaptotagmin antibodies (Fig. 3*E*), which showed a similar pattern of distribution in the presynaptic terminals.

Expression of class A–E Ca^{2+} channels in the dorsal horn

The dorsal horn of the spinal cord is the region where finely myelinated A-δ and unmyelinated C fiber afferents enter and terminate on interneurons that in turn make synaptic contacts with the motor neurons of the ventral horn (Jancs'o and Kiraly, 1980; Nagy and Hunt, 1983). Hence, we were interested in investigating the distribution of class A–E Ca^{2+} channels in the superficial laminae of the dorsal horn. Our results show that class A α_1 subunits are located primarily in nerve terminals in the dorsal horn (Fig. 4*A,B*). The highest density of staining is found in laminae 2–6, whereas the density of terminals containing α_{1A} in lamina 1 is much lower than in the deeper laminae (Fig. 4*A,B*; *arrows* denote the dorsal edge of the slice).

In contrast, anti-CNB2 staining for class B N-type Ca^{2+} channels is evenly distributed throughout all the laminae of the dorsal horn (Fig. 4*C*; *arrows* denote the dorsal edge of the slice). There is immunoreactivity for anti-CNB2 antibodies in nerve terminals

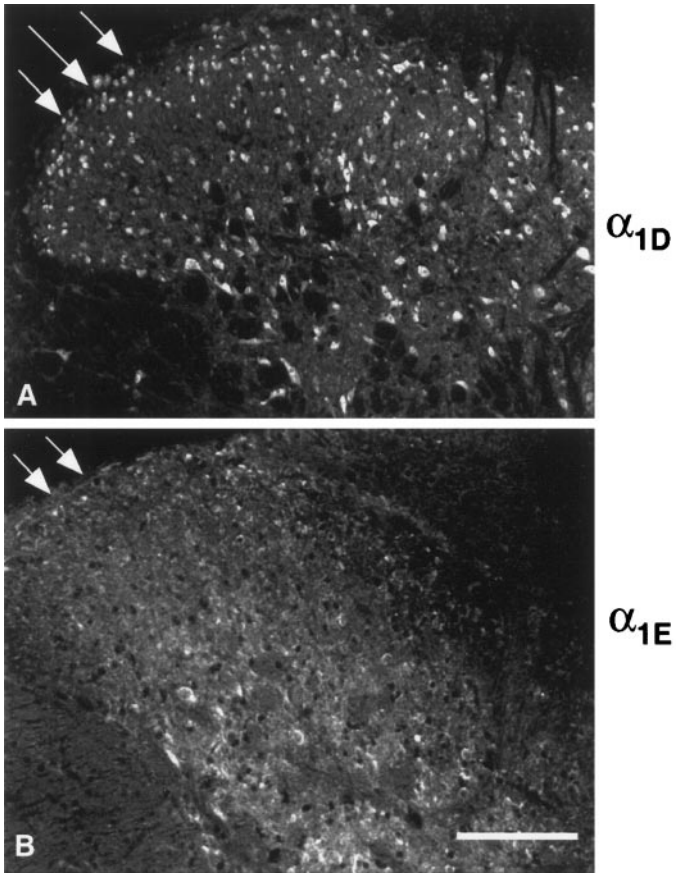

Figure 5. Localization of class D and class E Ca^{2+} channels in the dorsal horn. *A*, Tissue section labeled with anti-CND1 antibodies illustrating localization in the cell bodies found throughout the dorsal horn. *B*, Tissue section stained with anti-CNE2 antibodies demonstrating the presence of class E channels along the cell bodies of neurons. *Arrows* point to the dorsal surface of the spinal cord. Scale bar, 100 μm.

and cell bodies (Fig. 4*C,D*). The staining over the soma is both smooth and punctate in appearance (Fig. 4*D*), suggesting a low density of α_{1B} in the cell surface membrane and a higher density in nerve terminals forming synapses on the cell body.

Anti-CNC1 antibodies to class C L-type Ca^{2+} channels stained mainly the somata of cell bodies scattered throughout the entire dorsal horn (Fig. 4*E*). The staining over the cell surface was punctate in appearance and appeared to extend along the proximal portions of the dendrites (Fig. 4*F*). In hippocampal neurons, a similar pattern represents clusters of L-type Ca^{2+} channels in the postsynaptic membrane (Hell et al., 1993, 1996). Localization of class D and class E Ca^{2+} channels was mainly in the soma of neurons in the dorsal horn (Fig. 5, *A* and *B*, respectively). In both cases, there is smooth staining over the cell body surface and along the proximal dendrites. In the case of class E, there was also occasional punctate staining on the cell bodies and dendrites in the surrounding neuropil.

Colocalization of class A and B Ca^{2+} channels analyzed by double immunofluorescence

Double-labeling studies were performed to confirm the localization of class A and B Ca^{2+} channels at the NMJ and in nerve terminals in the spinal cord. To confirm the localization of class A Ca^{2+} channels in the NMJ, muscle sections were stained with anti-CNA1 antibodies and anti-synaptotagmin antibodies (Fig. 6*A*). We observed colocalization of these two proteins in nerve terminals, indicating the presence of class A Ca^{2+} channels at the NMJ of rats. Within the presynaptic terminals, there were regions of distinct staining for synaptotagmin (red) and for class A Ca^{2+} channels (green). Regions of overlap (yellow/orange) may be active zones in which the synaptic vesicles (detected by synaptotagmin) are in contact with the membrane where class A channels are localized. Double-label experiments using anti-CNB2 antibodies and anti-synaptotagmin antibodies also confirm the presence of class B Ca^{2+} channels in the presynaptic terminals of the NMJ (Fig. 6*B*).

Double-labeling experiments were performed to determine whether the α_{1A} and α_{1B} punctate staining observed in the ventral horn was associated with nerve terminals (Fig. 6*C,D*). Sections stained with anti-CNA1 (green) and anti-syntaxin (red) indicated that class A calcium channels are associated with nerve terminals (yellow) that form synapses with motor neurons (Fig. 6*C*). Likewise, tissue sections (Fig. 6*D*) incubated with anti-CNB2 (green) and anti-syntaxin (red) antibodies suggest that these two proteins are colocalized (yellow regions) in nerve terminals in the ventral horn of the spinal cord.

Our experiments with single-labeling procedures indicate that the distribution of nerve terminals containing α_{1A} and α_{1B} in the dorsal horn is complementary rather than overlapping. To look for nerve terminals containing both α_{1A} and α_{1B}, tissue sections were stained with anti-CNA1 (red) and anti-CNB2 antibodies (green) to investigate their distribution in the same nerve terminals at the transition zone between laminae I and II (Fig. 6*E*). In the superficial layers of the spinal cord, there are occasional terminals in which class A and class B Ca^{2+} channels are colocalized (Fig. 6*E*, *yellow*), but the staining patterns are primarily distinct, suggesting that most individual terminals in this region contain α_{1A} or α_{1B}, but not both.

In the dorsal horn of the spinal cord, double-labeling studies using anti-CNA1 antibodies (Fig. 7*A*, *green*) and anti-substance P antibodies (Fig. 7*B*, *red*) reveal that these two are only rarely localized in the same nerve terminals (Fig. 7*C*, *yellow*). Even when the transition zone between the staining for substance P and class A channels is examined at higher magnification, few nerve terminals are stained yellow, indicating little if any colocalization of substance P and α_{1A} (Fig. 7*G*). In contrast, comparison of the localization of the nerve terminals containing the α_1 subunit of class B Ca^{2+} channels (Fig. 7*D*, *green*) with the nerve terminals of primary afferent fibers containing substance P (Fig. 7*E*, *red*) shows a substantial overlap of the distribution of these nerve terminals (Fig. 7*F,H*, *yellow*). These results indicate that substance P is located in terminals that have N-type Ca^{2+} channels.

DISCUSSION

Ca^{2+} channels in motor neurons

Our results demonstrate that the various classes of Ca^{2+} channels have distinct patterns of distribution along the cell bodies, dendrites, and nerve terminals of spinal motor neurons that innervate skeletal muscles and suggest distinct functional roles for the different channel types. Whole-cell patch-clamp studies of embryonic spinal motor neuron cultures have demonstrated that these cells express Ca^{2+} channels that are sensitive to dihydropyridines, ω-conotoxin, and ω-agatoxin IVA along with a current that is resistant to these agents (Mynlieff and Beam, 1992; Hivert et al., 1995). Our findings are consistent with these studies, which

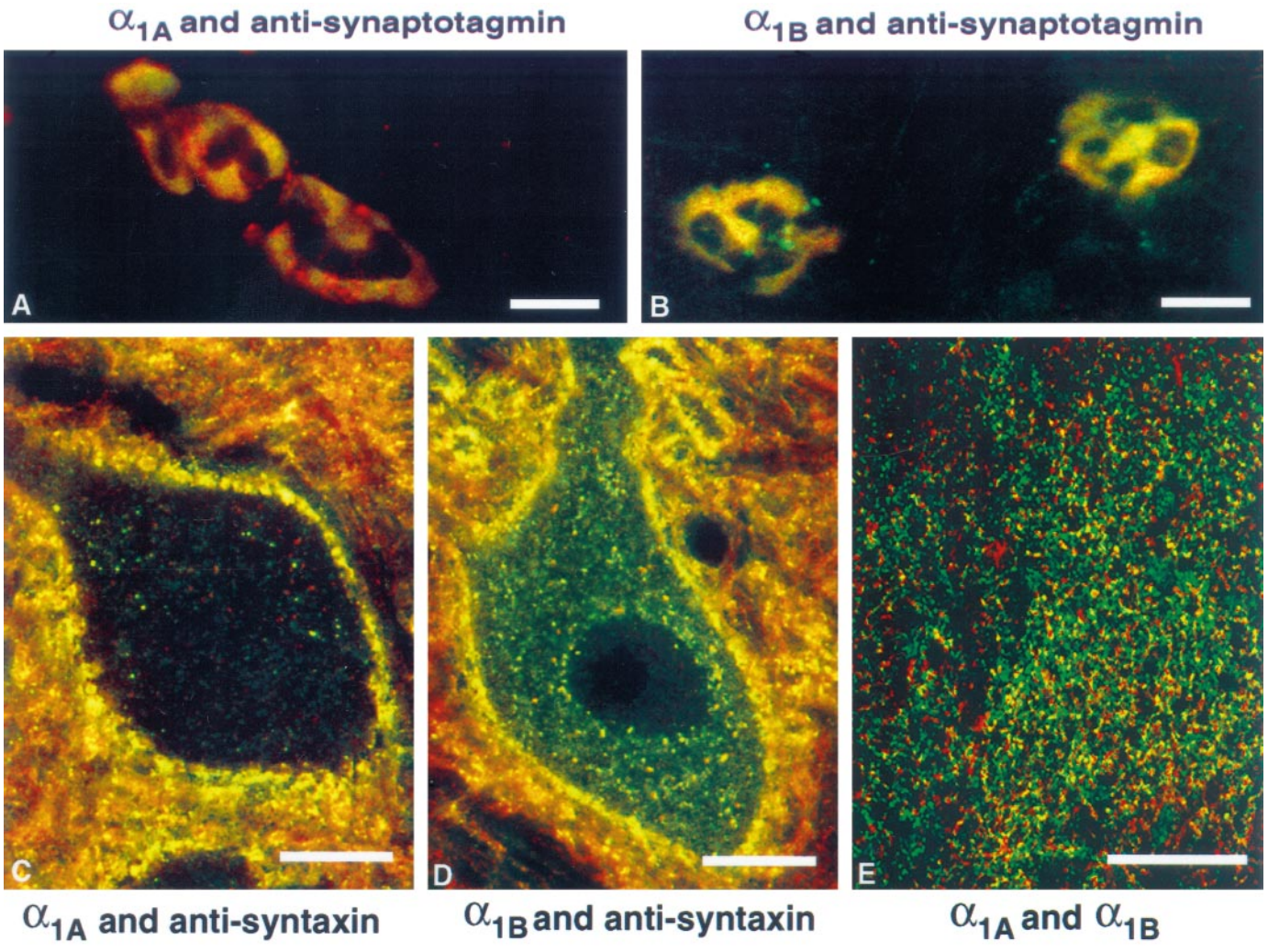

Figure 6. Colocalization studies at the NMJ and in spinal cord. *A*, Muscle tissue section incubated with anti-CNA1 (*green*) and anti-synaptotagmin (*red*) antibodies illustrating the presence of class A channels at the NMJ. Regions of *yellow* represent colocalization. *B*, Muscle tissue section labeled with both anti-CNB2 (*green*) and anti-synaptotagmin (*red*) antibodies demonstrating the presence of class B Ca^{2+} channels at the NMJ. Regions of *yellow* represent areas of colocalization. *C*, Tissue section from ventral horn double-labeled with anti-CNA1 (*green*) and anti-syntaxin (*red*) demonstrating colocalization (*yellow*) of these two proteins in terminals. *D*, Tissue sections from ventral horn were double-labeled with anti-CNB2 (*green*) and anti-syntaxin (*red*) to show colocalization in terminals (*yellow*). *E*, Section labeled with anti-CNA1 (*green*) and anti-CNB2 (*red*) antibodies illustrating the distribution of these terminals in the superficial layers of the dorsal horn. Areas of *yellow* represent colocalization of these two Ca^{2+} channels in terminals. The *top* of the section is dorsal. Laminae 2 and 3 are illustrated. Scale bars: *A, B*, 10 μm; *C–E*, 25 μm.

show that at least L-, N-, P-, and R-type Ca^{2+} currents are observed in the cell bodies of spinal motor neurons.

About half of the surface area of the cell body and three-fourths of the dendritic membrane of motor neurons is covered by synaptic boutons. The motor neuron receives excitatory input from the primary sensory neurons, excitatory and inhibitory inputs from interneurons that control motor function, and feedback inhibition from Renshaw and other inhibitory interneurons (Davidoff, 1983). In motor neurons, most inhibitory synapses are close to the cell body, whereas excitatory inputs are farther out on dendrites (Davidoff, 1983). With use of antibodies to class A and class B Ca^{2+} channels, our immunocytochemical studies show that both of these channel types are present in terminals that impinge on the cell body and dendrites, suggesting that these channels are present in both excitatory and inhibitory synapses in this region of the spinal cord.

Ca^{2+} channels at the NMJ

Neurotransmission at the frog NMJ is completely blocked by low concentrations of ω-conotoxin GVIA, and fluorescently tagged ω-conotoxin GVIA labels presynaptic nerve terminals, indicating that Ca^{2+} channels sensitive to this toxin are responsible for transmission at this synapse in the frog (Kerr and Yoshikami, 1984; Robitaille et al., 1990; Cohen et al., 1991; Tarelli et al., 1991). These results indicate that the amphibian equivalent of the class B N-type Ca^{2+} channels is responsible for neurotransmission at the NMJ.

The identity of the Ca^{2+} channels involved in synaptic transmission at mammalian NMJs is less clear. Several reports have demonstrated that nerve-stimulated transmitter release at mammalian NMJs is not blocked by ω-conotoxin GVIA (Sano et al., 1987; DeLuca et al., 1991; Protti et al., 1991), and several studies

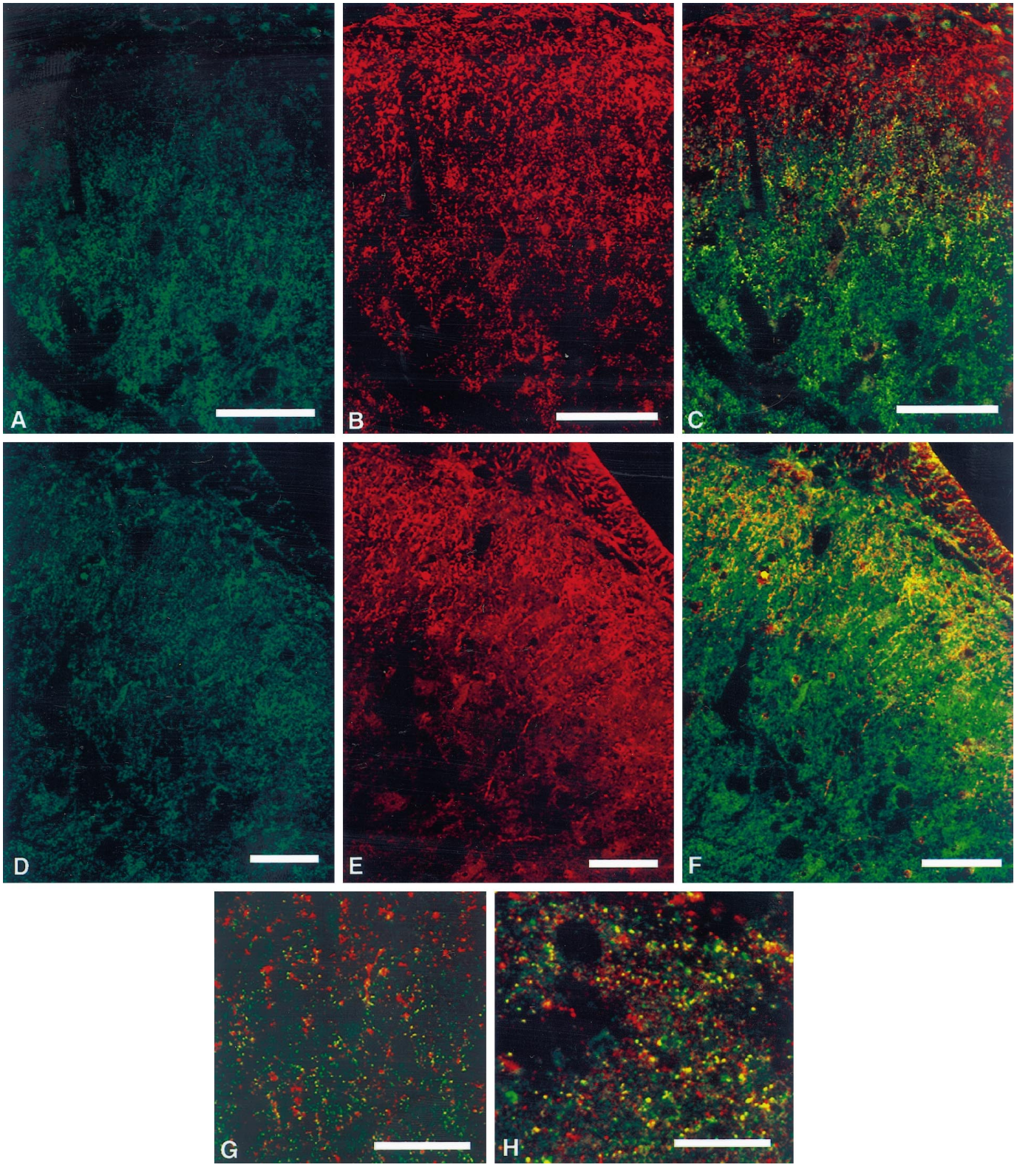

Figure 7. Colocalization studies in the spinal cord. *A*, Dorsal horn of spinal cord labeled with anti-CNA1 (*green*) antibodies. *B*, Same section in *A* that was also labeled with anti-substance P (*red*) antibodies. *C*, Merged image of *A* and *B* illustrating that in the dorsal horn colocalization between class A Ca^{2+} channels and substance P in terminals (*yellow regions*) is limited. *D*, Tissue section from the spinal cord labeled with anti-CNB2 (*green*). *E*, Same section as in *C*, double-labeled with anti-substance P (*red*) antibodies. *F*, Merged image of *C* and *D* illustrating that some terminals that are labeled with class B Ca^{2+} channels are also labeled with substance P (*yellow regions*). *G*, Higher magnification of merged image shown in *C* illustrating distribution of class A channels in the superficial portions of the dorsal horn (*red*), the distribution of substance P in this area (*green*), and regions of colocalization (*yellow*) of these two antibodies. *H*, Higher magnification of the image in *F* showing double-labeling with anti-CNB2 antibodies (*green*), anti-substance P antibodies (*red*), and terminals containing both proteins (*yellow*). Scale bars: *A–C*, 50 μm; *D–F*, 50 μm; *G, H*, 10 μm.

have indicated that the P/Q-type Ca^{2+} channels are the predominant ones that are involved in synaptic transmission at the mammalian NMJ (Llinas et., 1992; Uchitel et al., 1992; Protti and Uchitel, 1993; Bowersox et al., 1995; Sugiura et al., 1995). Our immunocytochemical studies support this conclusion, because the α_{1A} subunit is present in virtually all NMJs in the muscles we studied (diaphragm, tibialis anterior, gastrocnemius, and soleus). However, our results also show that N-type Ca^{2+} channels are present in a small fraction of nerve terminals in the tibialis anterior, soleus, and gastrocnemius muscles. Previous physiological studies of mammalian skeletal muscle have mainly used isolated diaphragm and phrenic nerve preparations and have observed that neuromuscular transmission is blocked by toxins that inhibit P/Q-type channels. Our experiments on various leg muscles, including the tibialis anterior muscle, demonstrate the presence of N-type channels at the NMJ as well. This finding may be related to the results of Rossoni et al. (1994) in which ^{125}I-ω-conotoxin GVIA was shown to bind to rat tibialis muscle end plates, and ω-conotoxin GVIA was capable of blocking neurotransmission both *in vitro* and *in vivo* in the tibialis anterior muscle. Physiological recordings of Ca^{2+} current in rat motor nerve terminals that innervate the extensor digitorum longus of the rat have also indicated the presence of N-type Ca^{2+} channels (Hamilton and Smith, 1992). Thus, P/Q-type Ca^{2+} channels containing α_{1A} subunits are the predominant Ca^{2+} channel at the rat NMJ, but nerve terminals with N-type Ca^{2+} channels containing α_{1B} are also present in some skeletal muscles.

Recent immunocytochemical experiments by Day et al. (1997) detected the presence of α_{1A}, α_{1B}, and α_{1E} staining in rat NMJ. After denervation, α_{1A} staining disappeared completely, whereas α_{1B} and α_{1E} staining did not. These results suggest that α_{1A} is exclusively localized in the presynaptic terminal but that α_{1B} and α_{1E} are also localized in Schwann cells (Day et al., 1997). In contrast, our results show the presence of class B channels in a subset of presynaptic terminals in rat leg muscles. Although the denervation study by Day et al. (1997) shows that N-type channels remain after nerve degeneration, it does not rule out the presence of some N-type channels in the presynaptic terminals of spinal motor neurons as well. Thus, both studies show that the predominant channel present at the rat NMJ is the P/Q-type channel, but our experiments also indicate that N-type Ca^{2+} channels may be present at some NMJs and that their presence is muscle dependent. We did not detect either α_{1B} or α_{1E} in most NMJs. This difference from the results of Day et al. (1997) may result from lower affinity of our anti-peptide antibodies compared with the anti-fusion protein antibodies used by Day et al. (1997), or from weak cross-reactivity of the antibodies of Day et al. (1997) with other proteins present in Schwann cells.

Ca^{2+} channels in the dorsal horn

The superficial dorsal horn of the spinal cord is involved in the processing of sensory information and forms the site of the first synapses in pain pathways. This region is the site of interaction of substance P, calcitonin gene-related peptide, and enkephalin, which have distinct regions of localization (Basbaum and Fields, 1984; Millan, 1986; Ruda et al., 1988; Villar et al., 1989). A functional relationship has been demonstrated between primary afferents that contain substance P and enkephalin-containing spinal interneurons (Basbaum and Fields, 1984; Millan, 1986; Ruda et al., 1988). In addition, several studies have demonstrated that primary nociceptive afferents release substance P (Brodin et al., 1987; Budai and Larson, 1996), whereas opiates have been shown to inhibit the release of substance P both *in vivo* and *in vitro* (Jessel and Iversen, 1977; Yaksh et al., 1980). Calcitonin gene-related peptide is known to be colocalized with substance P (Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984) and has been implicated in the modulation of nociception in the superficial laminae of the spinal cord (Wiesenfeld-Hallin et al., 1984; Kuraishi et al., 1988). Collectively, these studies implicate primary afferents that contain substance P as major contributors to pain pathways.

Our immunocytochemical studies suggest that N-type Ca^{2+} channels are the predominant Ca^{2+} channels associated with primary afferent fibers that contain substance P, whereas terminals containing class A P/Q-type Ca^{2+} channels are much fewer in number. In the formalin model of inflammation, N-type and P/Q-type but not L-type Ca^{2+} channels have been shown to be involved in the inflammation-evoked hyperexcitability of dorsal horn neurons after peripheral injection (Diaz and Dickenson, 1997). N-type Ca^{2+} channel antagonists have been shown to block substance P release from primary sensory neurons in culture (Holz et al., 1988). After formalin inflammation, ω-agatoxin IVA produced a strong dose-dependent reduction in the second phase of formalin response but no significant effect on the acute phase, suggesting that P/Q-type channels are involved only in the second phase of response (Diaz and Dickenson, 1997). With the predominance of class B N-type Ca^{2+} channels in laminae I and II of the dorsal horn compared with class A P/Q-type channels, our results and these electrophysiological studies suggest a primary role for class B N-type Ca^{2+} channels in initial pain responses in the dorsal horn of the spinal cord, with class A P/Q-type Ca^{2+} channels having a role in the second phase of response to inflammatory stimuli. These results provide a molecular basis for the selective block of pain stimuli by SNX-111, a synthetic analog of ω-conotoxin GVIA, which is under evaluation for control of neuropathic pain (Miljanich and Ramachandran, 1995).

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