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NEUROKININ RELEASE IN THE RAT NUCLEUS OF THE SOLITARY TRACT VIA NMDA AND AMPA RECEPTORS

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Abstract—Neurokinins (substance P, neurokinin A and neurokinin B) and the neurokinin receptors, the NK1 and NK3 receptors, are largely expressed in the nucleus of the solitary tract (NST) where they are involved in the central regulation of visceral function. Studying the mechanisms that control neurokinin release can provide valuable information concerning the control of autonomic functions subserved by the NST. Glutamate is the principal excitatory neurotransmitter in the NST and the main neurotransmitter of afferent vagal fibers. Neurokinins and glutamate may interact within the NST. In the present study, we have examined the contribution of the *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtypes of glutamate receptors on the release of the endogenous neurokinins in the NST. We used internalization of the NK1 or NK3 receptor as an index of endogenous neurokinin release assessed by immunocytochemical visualization of the NK1 or NK3 receptor endocytosis. Experiments were performed *in vitro* using rat brainstem slices. A first series of experiments were done in order to validate our *in vitro* preparation. Application of substance P, neurokinin A or neurokinin B induced dose-dependent internalization of NK1 and NK3 receptor. This was blocked by the endocytosis inhibitor, phenylarzine oxide. The NK1 receptor antagonist SR140333 blocked internalization of NK1 receptor induced by the three neurokinins. In addition, the internalization NK1 or NK3 receptor was reversible. These results demonstrate that internalization and recycling mechanisms of NK1 or NK3 receptor were preserved in *in vitro* brainstem slices. Application of NMDA or AMPA induced internalization of NK1 receptor. This was blocked by the application of SR140333 suggesting that NK1 receptor internalization is due to the binding of endogenous neurokinin released under the effects of NMDA and AMPA. Application of NMDA or AMPA had no effect on NK3 receptor. Application of tetrodotoxin blocked NK1 receptor internalization induced by NMDA, demonstrating that the release of neurokinins is dependent of axon potential propagation. This result excludes the hypothesis of a release on neurokinins via pre-synaptic NMDA receptors located on neurokinin-containing axon terminals. NMDA or AMPA may directly induce neurokinin release in the NST by acting on receptors located on the cell bodies and dendrites of neurokinin-containing neurons. Release of neurokinins may also be the result of a general activation of neuron networks of the NST by NMDA or AMPA.

To conclude, our results suggest that glutamate, through activation of post-synaptic NMDA and AMPA receptors, contributes to neurokinin signaling in the NST.

Key words: substance P, neurokinin A, neurokinin B, vagus nerve, glutamate.

The neurokinins substance P, neurokinin A, and neurokinin B are neuropeptides subserving a wide range of physiological functions (Otsuka and Yoshioka, 1993). Substance P displays high affinity for neurokinin-1 receptors (NK1 receptors) whereas neurokinin A and neurokinin B bind preferentially to neurokinin-2 receptors and neurokinin-3 receptors (NK3 receptors) respectively (Nakanishi, 1991). However, substance P, neurokinin A and neurokinin B may act on all three receptors (Harlan et al., 1989; Nakanishi, 1991; Hastrup and Schwartz, 1996; Wijkhuisen et al., 1999; Torrens et al., 2000). It is now well established that agonist binding induces internalization of neurokinin receptors and this internalization has been used as an index of neurokinin release in both central and peripheral nervous systems (Garland et al., 1994; Grady et al., 1995; Mantyh et al., 1995a,b; Garland et al., 1996; Liu et al., 1997; Marvizon et al., 1997; Mann et al., 1999; Jenkinson et al., 2000).

In the brainstem, the nucleus of the solitary tract (NST) plays a key role in central regulation of autonomic functions. Primary afferents from gustatory, gastrointestinal, cardiovascular, and respiratory organs, particularly those supplied by the vagus nerve, terminate within the NST (Sawchenko et al., 1987; Berthoud and Neuhuber, 2000). *In vitro* electrophysiological studies have demonstrated depolarizing effects of neurokinins on NST neurons (King et al., 1993; Maubach and Jones, 1997). Several studies have shown that the NST is enriched with axon terminals containing substance P, neurokinin A and neurokinin B (Kalia et al., 1984;

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Abbreviations: ACF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DL-APV, DL-2-amino-5-phosphonovaleric acid; NK1 receptor, neurokinin-1 receptor; NK3 receptor, neurokinin-3 receptor; NMDA, *N*-methyl-D-aspartate; NST, nucleus of the solitary tract; PB, phosphate buffer; TTX, tetrodotoxin.

Baude et al., 1989; Lucas et al., 1992; Marksteiner et al., 1992; Saha et al., 1995a). Neurokinins can originate from peripheral nerves, central brain structures, or the NST itself. Afferent trigeminal fibers (Sugimoto et al., 1997) and a few afferent vagal fibers (Sykes et al., 1994) projecting to the NST contain substance P. Substance P-containing afferent fibers project from the raphe to the NST (Thor and Helke, 1989). In addition, substance P and/or neurokinin A-containing neurons are present within the NST (Ljungdhal et al., 1978; Harlan et al., 1989). The source of neurokinin B-containing fibers is unknown. Binding sites for substance P, neurokinin A and neurokinin B have been described in the NST (Mantyh et al., 1989) and mRNAs coding for NK1 and NK3 receptors as well as the proteins themselves have been detected (Nakaya et al., 1994; Carpentier and Baude, 1996; Ding et al., 1996; Baude and Shigemoto, 1998; Dixon et al., 1998). Considerable evidence has implicated neurokinins and their receptors in control of cardiovascular, digestive, gustatory and respiratory function by the NST (Improta and Broccardo, 1990; King et al., 1993; Lawrence and Jarrott, 1996; Mazzone and Geraghty, 2000; Otsuka and Yoshioka, 1993). Therefore, studying the mechanisms that control neurokinin release can provide valuable information concerning the control of vital functions subserved by the NST.

Glutamate acts as an excitatory neurotransmitter in the NST. It is the main neurotransmitter of afferent vagal fibers (Saha et al., 1995b; Schaffar et al., 1997; Sykes et al., 1997). Both *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5 methyl-4-isoxazole propionic acid (AMPA) receptors mediate glutamatergic neurotransmission in the NST (Kessler and Jean, 1991; Tell and Jean, 1993; Aylwin et al., 1997; Zhang and Mifflin, 1998; Yen et al., 1999). In addition, mRNAs coding for the different subunits of NMDA and AMPA receptors have been detected in NST neurons (Watanabe et al., 1994; Guthmann and Herbert, 1999) as well as the proteins themselves (Ambalavanar et al., 1998; Aicher et al., 1999; Guthmann and Herbert, 1999; Kessler and Baude, 1999).

In the spinal cord, it has been suggested that glutamate through NMDA receptors may regulate the release of substance P from primary afferents (Liu et al., 1994, 1997; Marvizon et al., 1997). In the NST, vagal afferents have been shown to contain NMDA receptors (Aicher et al., 1999). In addition, glutamate and substance P (or neurokinin A) co-exist in axon terminals (Saha et al., 1995a) of the NST, suggestive of co-release. In the present study, we have investigated in the NST whether glutamate, via NMDA and AMPA receptors, is involved in the release of the neurokinins assessed by immunocytochemical visualization of the internalization NK1 or NK3 receptor in rat brainstem slices.

EXPERIMENTAL PROCEDURES

Preparation and treatment of brainstem slices

All experimental procedures were designed to minimize animal suffering in compliance with the European Community Council directive (86/609/EEC) concerning animal experimentation. Four to 10 rats (100–250 g) were used for each treatment. Immediately after induction of anesthesia with halothane, rats were decapitated, brainstems dissected out, and transverse slices (300 µm thick) made using a vibratome (Leica). Slices were immediately incubated at 32°C in artificial cerebrospinal fluid (ACF, pH 7.3) containing in mM: 125 NaCl, 5 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$ and 10 glucose, bubbled with 95% 0_2 and 5% CO_2 for at least 1 h prior to drug application.

Drugs were diluted in ACF including substance P (0.1 µm or 5 µm, Sigma), neurokinin A (0.1 µM or 10 µM, Sigma), neurokinin B (0.1 µM or 10 µM, Sigma), NMDA (100 µM, Sigma), AMPA (50 µM, Sigma), phenylarsine oxide (10 µM, Sigma), tetrodotoxin (TTX, 1 µM, Sigma), and DL-2-amino-5-phosphonovaleric acid (DL-APV, 100 µM, Sigma). Neurokinin concentrations used in this study were at least 100 times higher than the K_d for the respective preferred receptors (Helke et al., 1990). Substance P, neurokinin A or neurokinin B were generally applied for 15 min followed or not by rinsing in ACF 60 min. In some cases, two 15-min applications of substance P or neurokinin B were separated by rinsing in ACF for 60 min. NMDA application lasted for 2 min and slices were then kept in ACF for 15 min. Pretreatment with SR140333 (5 µM, Neosystem), an NK1 receptor selective antagonist (Edmonds-Alt et al., 1993), lasted for 30 min prior to application of either substance P or neurokinin A (15 min), or NMDA or AMPA (2 or 5 min respectively, followed by immersion in SR140333 for 15 min). Pretreatment with DL-APV, a specific antagonist of NMDA receptors lasted for 30 min prior to application of NMDA (2 min followed by immersion in DL-APV for 15 min). Pretreatment with phenylarsine oxide, and endocytosis inhibitor, lasted for 5 min, then slices were rinsed briefly three times in ACF prior to application of neurokinins (15 min). Pretreatment with TTX, a blocker of Na^+ channels that inhibits action potential propagation, prior to application of either NMDA (2 min, followed by immersion in TTX for 15 min) or substance P (15 min), lasted for 30 min. After drug treatment, slices were fixed overnight at 4°C in 4% paraformaldehyde diluted in phosphate buffer (PB; 0.1 M, pH 7.4), immersed in PB supplemented with 30% sucrose for 4 h at room temperature, and cut into sections (30 µm) with a cryotome.

As a control some animals were perfused through the aorta with 500 ml of a fixative solution containing 4% paraformaldehyde in PB after having been deeply anesthetized with a ketamine (50 mg/ml) and xylazine (7.5 mg/ml) solution at a dose of 2 ml/kg (i.m.). The medulla was removed and cut into sections (50 µm) with a vibratome (Leica).

Immunofluorescence

Sections obtained from brainstem slices or from brainstem of perfusion-fixed rats were collected in PB saline (PBS, PB containing 0.9% NaCl) and incubated for 1h at room temperature in blocking serum (5% normal goat serum in PBS). Next the sections were incubated overnight at 4°C in primary antibodies directed to NK1 receptor or NK3 receptors diluted (0.5 µg/ml) in PBS containing 0.3% Triton X-100, 1% normal goat serum, and 0.01% sodium azide. After three washes, sections were incubated for 1 h at room temperature in secondary antibody goat anti-rabbit IgG coupled either to oregon green (Molecular Probes) or to CY^3 (Jackson Immunoresearch) diluted 1:200 in PBS and 0.3% Triton X-100. After incubation, sections were washed three times with PBS and mounted with a solution containing 50% PB and 50% glycerol and 0.01 sodium azide. In control tests performed without the primary antibody, no immunoreactivity resembling specific labeling was observed. Primary antibodies used were generously donated by Dr. R. Shigemoto. They are polyclonal rabbit antibodies directed against the C-terminal amino-acid residues 349–407 of NK1 receptor protein and 388–452 of NK3 receptor protein. Production, purification and characterization of the antisera have been described elsewhere (Shigemoto et al., 1993; Ding et al., 1996).

Immunoreactivity was analyzed with a confocal Leica TCS

SP2 microscope using an argon laser as the excitation source (488 nm) and an emission filter band pass (525 nm) for oregon green, or an helium/neon laser as the excitation source (543 nm) and an emission filter band pass (620 nm) for CY^3. The pinhole size was adjusted for maximum resolution along the depth axis with the lenses and wavelengths used. Series of digitized optical sections (512×512 pixels; step: 0.5–1 μm; lens: ×20, ×40 or ×63; scanning zoom: 1–4) were collected and maximum intensity projections were derived using Leica TCS software. Images were prepared for printing with a final resolution of at least 150 dpi using Adobe Photoshop software. Only image contrast and brightness were digitally adjusted.

RESULTS

In order to validate the effects of NMDA and AMPA, we have performed a first series of experiments to ascertain whether internalization of NK1 and NK3 receptors were effective in our *in vitro* preparations. We then tested whether NMDA and AMPA could induce the internalization of NK1 or NK3 receptor.

NK1 and NK3 receptors immunoreactivity in brainstem slices treated with neurokinins

On the sections obtained from the perfusion-fixed rat, NK1 and NK3 receptors immunoreactivity (Fig. 1A, B) was visible as a dense network of labeled dendrites and cell bodies in the NST and the adjacent dorsal motor nucleus of the vagus nerve. The NST is composed by several subnuclei; the medial subnucleus contain both immunoreactivity for NK1 and NK3 receptors. Most of the views shown in the present study are taken from the medial subnucleus all over the rostro-caudal extent of the NST. Immunoreactivity for NK1 or NK3 receptor was present on the surface of dendrites and cell bodies of neurons, and no labeling was visible within their cytoplasm (Fig. 1C, D). On the sections obtained from control *in vitro* brainstem slices, immunoreactivity for NK1 receptor (Fig. 2A, A1) and NK3 receptors (Fig. 3D) was found outlining the plasma membrane of cell bodies and dendrites of all labeled neurons. Together these results show that the internalization of NK1 and NK3 receptors in NST we later observed, was not an artifact of the slicing procedure.

Incubation of slices with exogenous neurokinins induced dramatic changes in receptors distribution. After incubation of slices with substance P (0.1 μM, 15 min; Fig. 2B, B1), neurokinin A (0.1 μM, 15 min; Fig. 3A) or neurokinin B (10 μM; Fig. 3C), immunoreactivity for NK1 receptor was no longer associated with the surface of neurons, but was visible as intensely immunoreactive intracellular spots within the cell bodies and dendrites of all labeled neurons in the NST (Fig. 2B1). Similar changes were visible for NK3 receptor immunoreactivity after incubation of slices with neurokinin B (1 μM, 15 min; Fig. 3E), neurokinin A (10 μM, 15 min) or substance P (5 μM). When brainstem slices were pretreated with the blocker of endocytosis, phenylarsine oxide, prior to application of exogenous neurokinins, immunoreactivity for neurokinin receptors (Fig. 2C) remained localized at the neuronal membrane, and no intracytoplasmic immunoreactive spots were visible. This demonstrates that the changes of the NK1 or NK3 receptor distribution was due to their internalization. Internalization of NK1 receptor induced by either substance P or neurokinin A was prevented by pretreatment of the brainstem slices with SR140333, a NK1 receptor antagonist (Figs. 2D and 3B). SR140333 by itself did not induce NK1 receptor internalization (Fig. 2E). The internalization of NK1 and NK3 receptors is dependent on the concentration of exogenous agonists applied to brainstem slices. Substance P (0.1 μM) induced internalization of NK1 receptor, but had no effect on NK3. Similarly, neurokinin B (0.1 μM) induced internalization of NK3 receptor but had no effect on NK1 receptor. Neurokinin A (0.1 μM) induced internalization of NK1 receptor, but had little effect on NK3 receptor. Increasing the concentrations of exogenously applied neurokinins (substance P up to 5 μM, and neurokinin A and neurokinin B up to 10 μM) induced internalization of both NK1 (Fig. 3C) and NK3 receptors. Internalization of NK1 and NK3 receptors was reversible. When application of substance P (0.1 μM, 15 min) or neurokinin B (0.1 μM, 15 min) was followed by rinsing in ACF for 60 min, immunoreactivity for NK1 or NK3 receptor was associated with the surface of neurons, and no intracytoplasmic punctate labeling could be seen (Fig. 3F, H). When two 15-min applications of substance P or neurokinin B separated by 60 min rinsing in ACF were performed, intracytoplasmic spots reappeared in cell bodies and dendrites (Fig. 3G, I).

Together, these results indicate that internalization and recycling mechanisms of NK1 and NK3 receptors were functional in brainstem slices. No internalization was seen in control sections demonstrating that internalization is the result of the application of the different drugs.

Immunoreactivity for NK1 and NK3 receptors on brainstem slices treated with NMDA and AMPA

Application of NMDA (100 μM, 2 min) induced internalization of NK1 receptor in cell bodies and dendrites of neurons of the NST (Fig. 4A–C) but had little or no effect on neurons within the adjacent dorsal motor nucleus of the vagus nerve (Fig. 4D). Internalization of NK1 receptor was demonstrated by the presence of immunoreactive intracytoplasmic spots in dendrites and cell bodies (Fig. 4C). SR140333 blocked the NMDA-induced internalization of NK1 receptor (Fig. 4E). Application of TTX (1 μM) prior to NMDA treatment blocked NK1 receptor internalization (Fig. 4F), but not that induced by substance P (Fig. 4G). The use of the NMDA receptors antagonist, DL-APV (100 μM), blocked the NMDA-induced NK1 receptor internalization (Fig. 5A, B). AMPA (50 μM, 5 min) induced internalization of NK1 receptor in cell bodies and dendrites in the NST (Fig. 5C, D), without visibly affecting those of the dorsal motor nucleus of the vagus nerve (Fig. 5E). NK1 receptor internalization induced by AMPA was blocked by SR140333 (Fig. 5F).

Application of either NMDA or AMPA had no effect on the distribution of NK3 immunoreactivity; no internalization of NK3 receptor was observed.

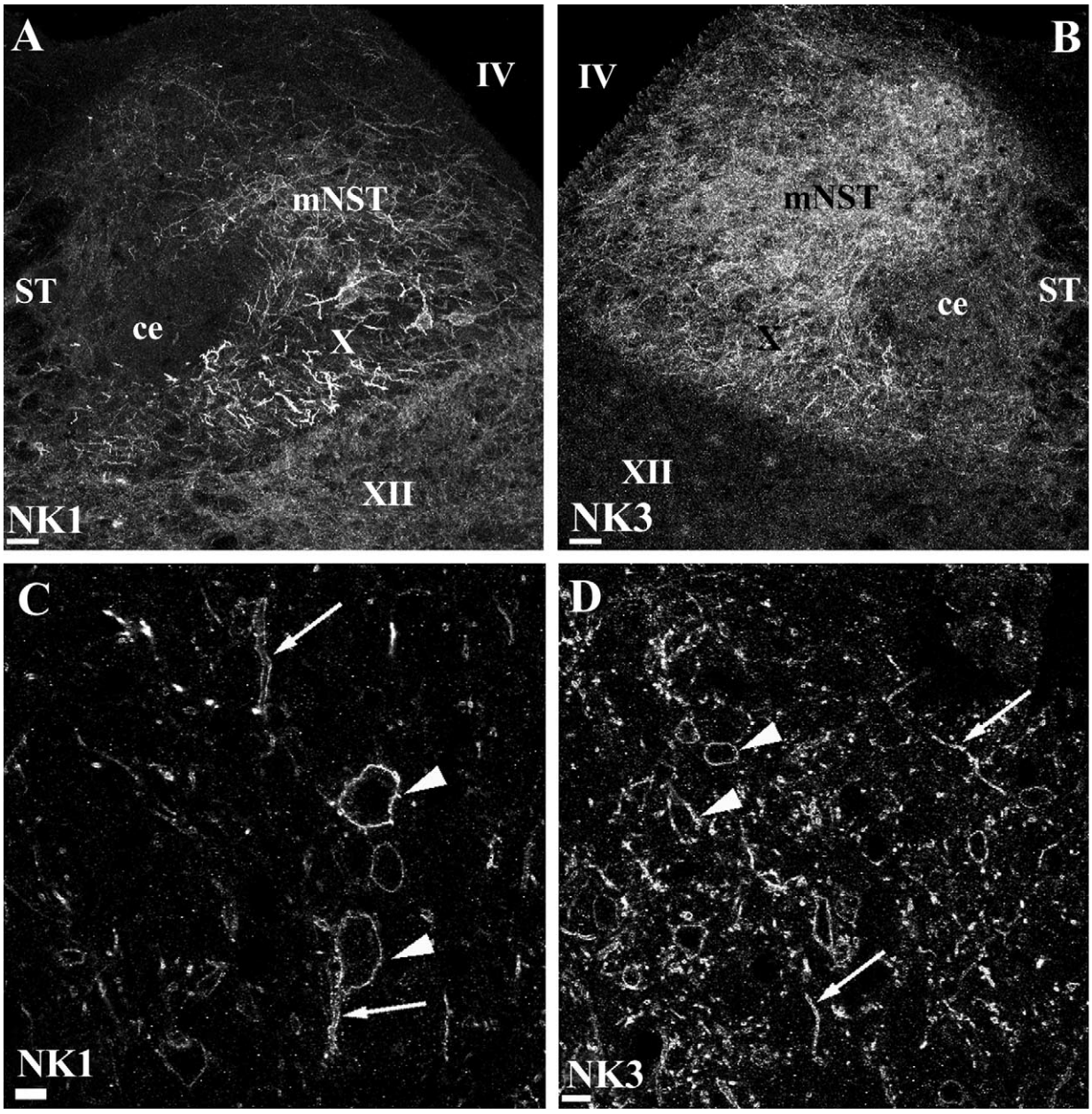

Fig. 1. Distribution of the immunoreactivity for NK1 (A, C) or NK3 (B, D) in the NST on brainstem sections obtained from perfusion-fixed rats. (A, B) NK1 or NK3 receptor immunoreactivity is present as a dense network of labeled cell bodies and dendrites. Note that the central subnucleus of the NST (ce) is weakly labeled for both receptors in comparison of the medial subnucleus of the NST (mNST). (C, D) NK1 or NK3 receptor immunoreactivity is present on the surface of cell bodies (arrowheads) and dendrites (arrows); no intracytoplasmic labeling is seen. Scale bars: in A and B = 45 µm; in C and D = 10 µm. ST, Solitary tract; IV, forth ventricle; X, dorsal motor nucleus of the vagus nerve; XII, motor nucleus of the hypoglossal nerve.

DISCUSSION

In the present study we have used the internalization of NK1 and NK3 receptors as an index to investigate the mechanisms through which NMDA and AMPA receptors may regulate release of endogenous neurokinins in the NST. We have performed *in vitro* experiments and we have first confirmed that the mechanisms of internalization and recycling of neurokinin receptors were preserved in brainstem slices. These control experiments support our results that provide evidence for a glutamate-induced release of endogenous neurokinins through activation of post-synaptic NMDA and AMPA receptors in the NST.

In vitro application of neurokinins induced internalization of NK1 and NK3 receptors in NST neurons

In line with previous results obtained in different areas of the central or peripheral nervous system (Mantyh et al., 1995a,b; Liu et al., 1997; Mann et al., 1999; Jenkinson et al., 2000; Marvizon et al., 1997), applica-

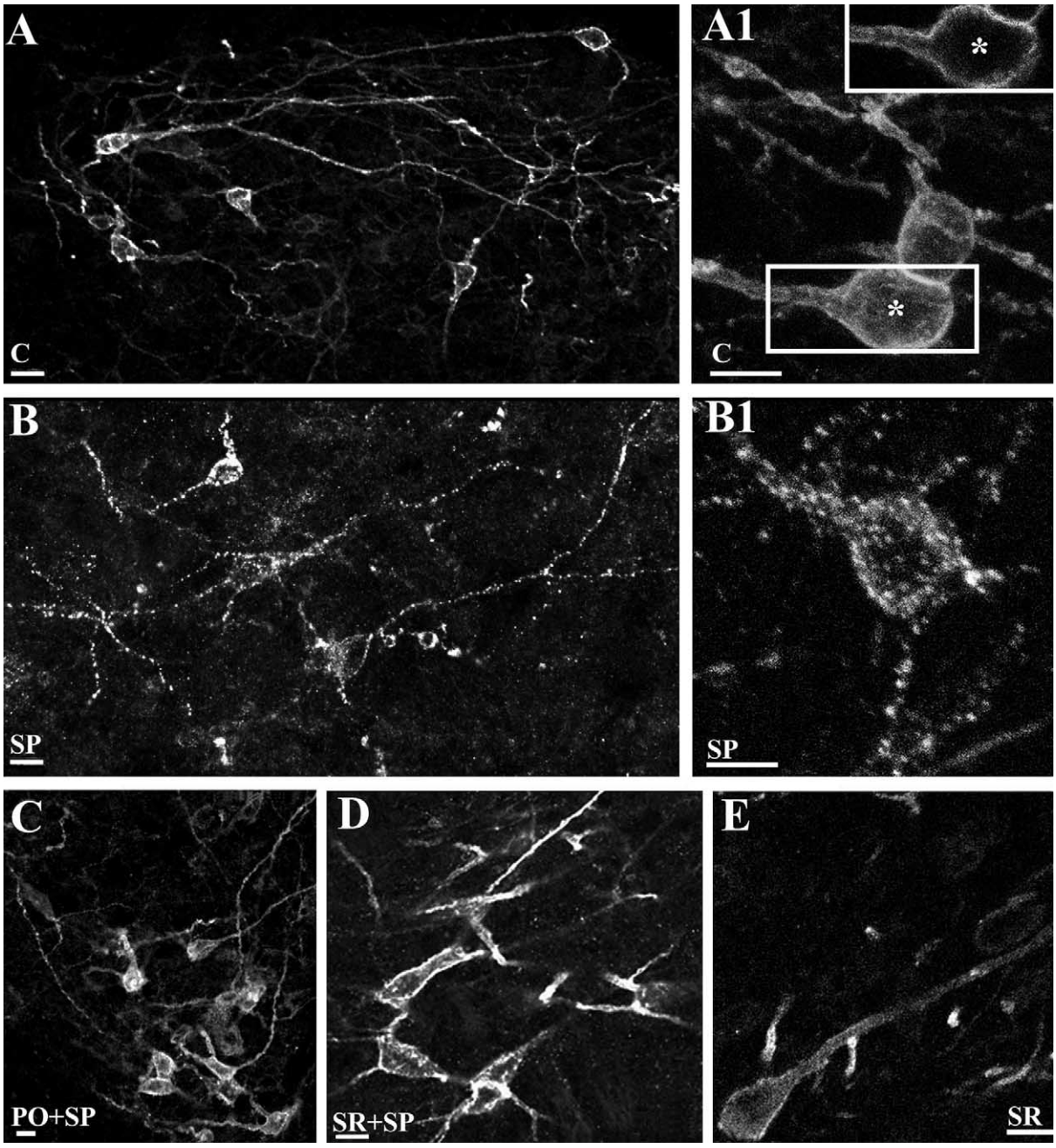

Fig. 2. Application of exogenous substance P (SP) to *in vitro* brainstem slices induces NK1 internalization in neurons of the NST. (A–A1) NK1 receptor immunoreactivity is located on the surface of neurons in control brainstem slices (c). It is clear from the insert in A1 showing a single optical section that no immunoreactivity is present within the cytoplasm. (B–B1) Incubation for 15 min in substance P (0.1 μM) induces dramatic changes in the distribution of the NK1 receptor immunoreactivity that is now visible as intracytoplasmic spots throughout cell bodies and dendrites (B1), suggesting endocytosis of NK1 receptor. (C) Application of phenylarzine oxide (PO, 1 μM), an inhibitor of endocytosis, blocks the SP-induced NK1 receptor internalization; NK1 receptor immunoreactivity remains located to the surface of neurons. (D) Incubation with the NK1 receptor antagonist, SR140333 (SR, 5 μM), blocks SP-induced NK1 receptor internalization; NK1 receptor immunoreactivity is visible on the surface of neurons. (E) By itself SR14033 has no effect on the distribution of the NK1 receptor immunoreactivity. Scale bars = 10 μm.

tion of exogenous substance P on brainstem slices induces NK1 receptor internalization in the NST. NK1 receptor undergoes rapid internalization upon activation, thus NK1 receptor internalization provides a measure of the functional consequences of neurokinin release, allowing one to assess when neurons expressing NK1 receptor are activated *in vivo* (Abbadie et al., 1997; Mantyh et al., 1995a,b; Trafton et al., 1999). Therefore, our results suggest that endogenously released substance P, neurokinin A or neurokinin B can activate NK1 receptor that are present on NST neurons (Baude and Shigemoto, 1998) but also those present on vagal efferent neurons of the dorsal motor nucleus of the vagus nerve (Blondeau et al., 2002). The selective NK1 receptor antagonist

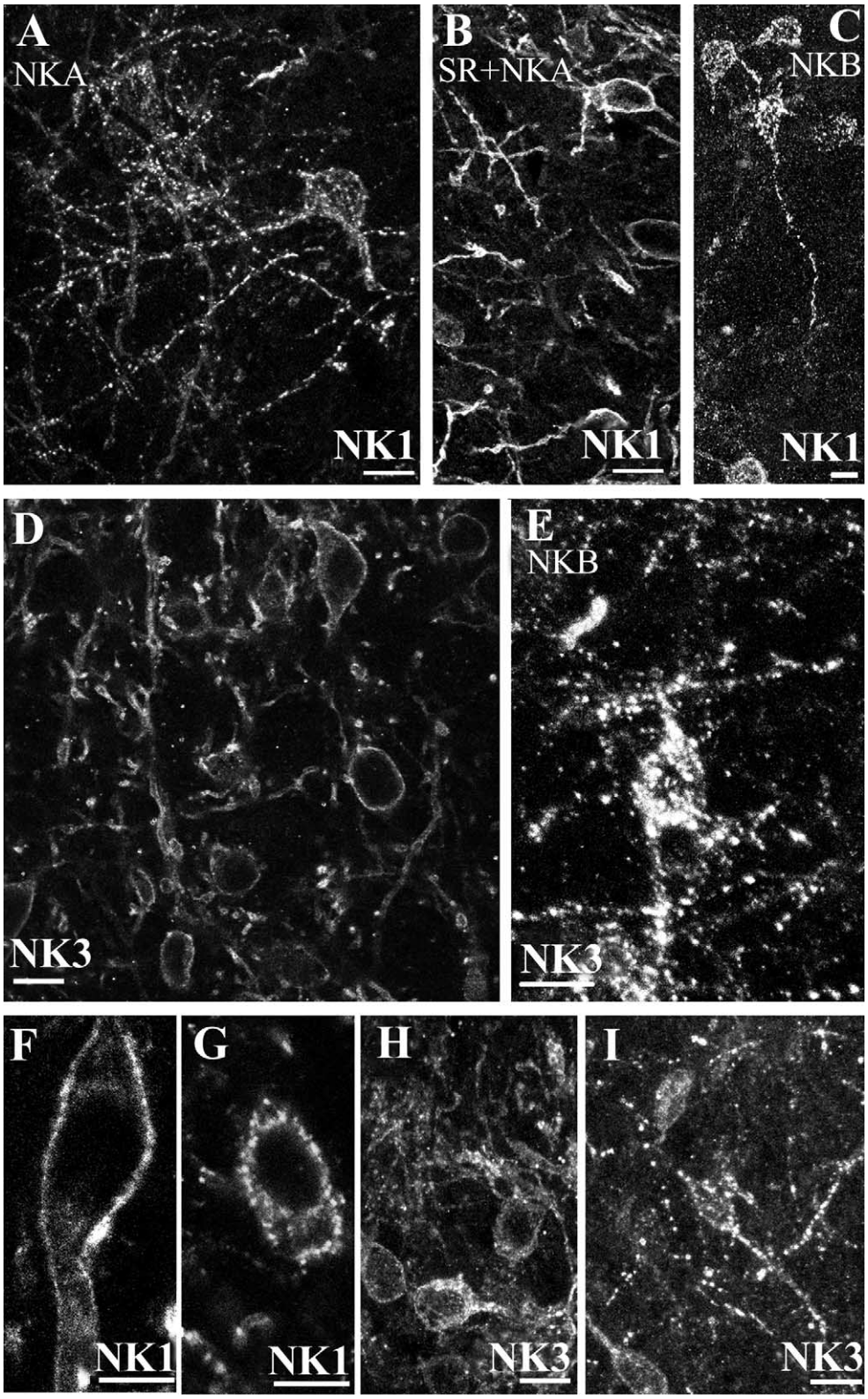

Fig. 3. Internalization of NK1 or NK3 in neurons of the NST after application of exogenous neurokinins. (A) Application of exogenous neurokinin A (NKA, 0.1 μM) induces internalization of the NK1 receptor. (B) SR140333 (SR, 5 μM) blocks the NK1 receptor internalization induced by NKA. (C) Application of exogenous neurokinin B (NKB, 10 μM) induces the internalization of NK1 receptor. (D) NK3 receptor immunoreactivity is located on the surface of neurons in control brain slices. (E) Application of exogenous neurokinin B (0.1 μM) induces internalization of NK3 receptor; NK3 receptor immunoreactivity is visible as intracytoplasmic spots in cell bodies and dendrites. The internalization NK1 or NK3 receptor is reversible in *in vitro* brainstem slices. (F, H) Experiments where the slices are incubated in either SP (F) or NKB (H) followed by rinsing for 60 min, immunoreactivity for NK1 or NK3 receptor is present on the surface of neurons. (G, I) Re-incubation in SP (G) or in NKB (I) induces internalization of the NK1 (G) or NK3 (I) receptors. Scale bars = 10 μm.

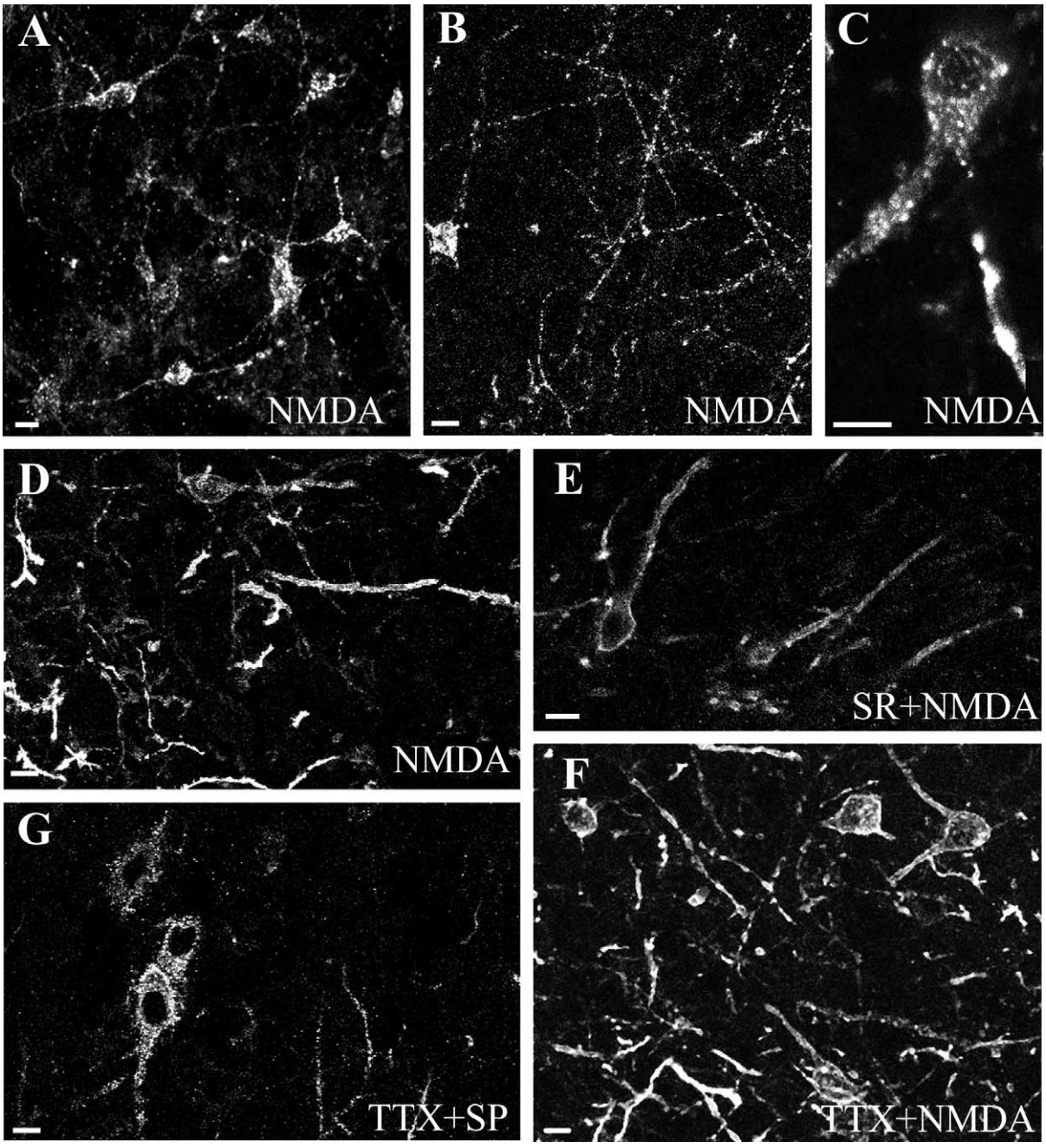

Fig. 4. NMDA induces internalization of NK1. (A, B) NMDA (100 μM) induces internalization of NK1 receptor in neurons of the medial subnucleus (A) and rostral subnucleus (B) of the NST. (C) Intracytoplasmic spots immunoreactive for NK1 receptor are present in the cell body and the dendrites of a neuron after the internalization of NK1 receptor. (D) In contrast, NMDA application does not visibly affect the surface distribution of the immunoreactivity for NK1 receptor in the neurons of the dorsal motor nucleus of the vagus nerve. (E) SR140333 (SR) blocks the NMDA-induced internalization of NK1 receptor. (F) Pretreatment with TTX (1 μM) prevents the NMDA-induced internalization of NK1 receptor. (G) Inversely, TTX application does not affect NK1 receptor internalization induced by substance P (SP). Scale bars = 10 μm.

SR14033 blocks the NK1 receptor internalization suggesting a direct action of neurokinin A on NK1 receptor. Previous studies have demonstrated that neurokinin A is a high-affinity ligand for NK1 receptor (Hastrup and Schwartz, 1996; Wijkhuisen et al., 1999; Torrens et al., 2000) and that it acts via NK1 receptor to depolarize NST neurons (Maubach and Jones, 1997). In addition, neurokinin B may also interact with NK1 receptor as high concentrations of neurokinin B can induce NK1 receptor internalization. A similar parallel between internalization and activation of NK3 receptor has not been clearly established. Nevertheless, substance P, neurokinin A and neurokinin B at the concentrations used here, induce internalization of NK3 receptor in NST neurons. Our results showing that neurokinin A induced NK3 receptor internalization are in line with previous results obtained in myenteric plexuses (Jenkinson et al., 2000).

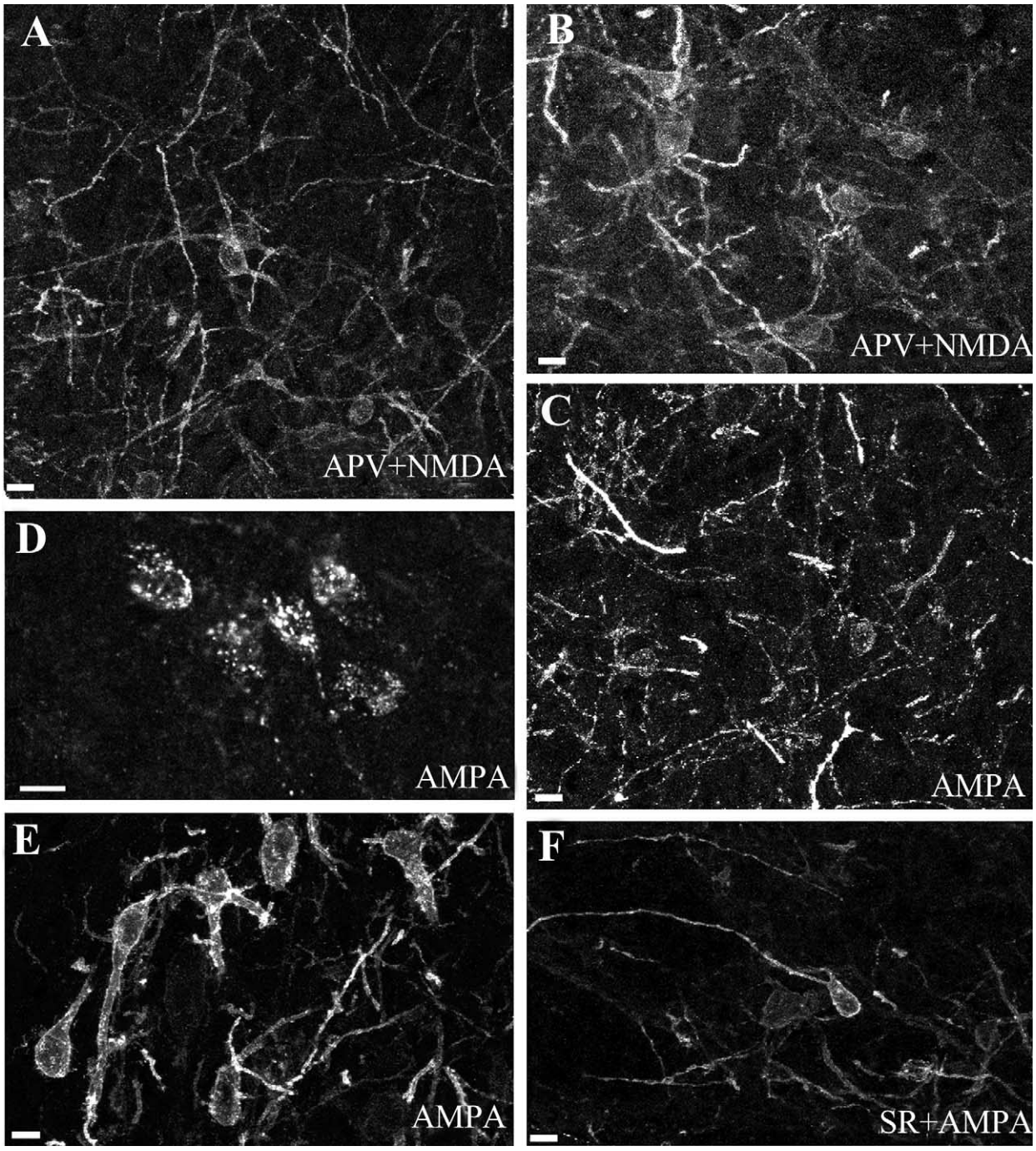

Fig. 5. NMDA and AMPA induce internalization of NK1. (A, B) Pretreatment with the NMDA receptors antagonist, DL-APV, prevents NMDA-induced internalization of NK1 receptor in neurons of the medial subnucleus (A) and rostral subnucleus (B) of the NST. (C) AMPA (50 μM) induces NK1 receptor internalization in neurons of the NST. (D) Intracytoplasmic spots immunoreactive for NK1 receptor are present in cell bodies and dendrites of neurons after NK1 receptor internalization. (E) AMPA application does not visibly affect the surface distribution of the immunoreactivity for NK1 receptor in the neurons of the dorsal motor nucleus of the vagus nerve. (F) Pretreatment with the NK1 receptor antagonist, SR140333 (SR), prevents AMPA-induced internalization of NK1 receptor in neurons of the NST. Scale bars = 10 μm.

Post-synaptic effects of NMDA and AMPA on neurokinins release in the NST

Application of NMDA to NST neurons in brainstem slices induces internalization of NK1 receptor which is blocked by the NMDA receptor antagonist, DL-APV, formally excluding any direct effect of NMDA on the NK1 receptor. Studies monitoring internalization of spinal NK1 receptor, as a measure of substance P release, have suggested that activation of pre-synaptic NMDA glutamate receptors facilitate the release of substance P from primary afferents which also co-localize glutamate (Liu et al., 1997; Marvizon et al., 1997). A similar hypothesis could be made for NST and is supported by

several data. First the neuropil of the NST is particularly rich in both substance P- and neurokinin A-containing fibers, some of which co-localize glutamate (Kalia et al., 1984; Baude et al., 1989; Saha et al., 1995a). Second, the presence of pre-synaptic NMDA receptors on axon terminals in the rat NST has been clearly established (Aicher et al., 1999). However, our result showing that NMDA induced-internalization of NK1 receptor is blocked by TTX, jeopardizes the above hypothesis. This result clearly indicates that the release of endogenous substance P or other neurokinins requires axon potential propagation in the NST. The data obtained in the spinal cord, showing a relation between NMDA receptors and substance P release, could be linked to the particular feature of noxious primary afferent which contained both substance P and glutamate (Battaglia and Rustioni, 1988) in addition to pre-synaptic NMDA receptors (Liu et al., 1994; Aicher et al., 1997). The vagal afferents represent the principal contingent of primary afferents in the NST. Similar to spinal primary afferents, vagal primary afferents are glutamatergic (Saha et al., 1995a,b; Schaffar et al., 1997; Sykes et al., 1997) and some contain pre-synaptic NMDA receptors (Aicher et al., 1997), but very few of them contain substance P in the rat (Sykes et al., 1994).

Release of endogenous neurokinins inducing the internalization of NK1 receptor in NST neurons in brainstem slices is not specific to NMDA as it can also be evoked by AMPA, another ionotropic glutamate receptor agonist. This is strengthened by the fact that SR140333 blocked the NK1 receptor internalization induced not only by NMDA but also by AMPA. This suggests that NK1 receptor internalization is due to the binding of endogenous neurokinins that have been released within brainstem slices under the effects of NMDA and AMPA. We have shown that the NMDA-induced internalization of NK1 receptor in the NST is blocked by pretreatment with TTX. In addition, no pre-synaptic AMPA receptors have been detected in the NST (Ambalavanar et al., 1998; Kessler and Baude, 1999; Lacassagne and Kessler, 2000). Altogether these results indicate that the action of NMDA and AMPA is mediated through post-synaptic receptors. A first hypothesis is that NMDA and/or AMPA induce release of neurokinins through the activation of post-synaptic NMDA receptors located on the soma and dendrites of substance P/neurokinin A-containing NST interneurons (Harlan et al., 1989; Ljungdhal et al., 1978). A second hypothesis, which does not exclude the first, would be that NMDA and/or AMPA indirectly induce neurokinin release through a general activation of neuron networks within the NST.

Neither NMDA nor AMPA induces the internalization of NK3 receptor in NST neurons. We have shown that internalization of NK1 or NK3 receptor by substance P, neurokinin A or neurokinin B is dose-dependent. Therefore, the concentration of neurokinin released by NMDA or AMPA might be too low to induce internalization of NK3 receptor. The failure of AMPA and NMDA to induce NK3 internalization can also be explained by the fact that neurokinin B, the preferential agonist of NK3, is not expressed by NST neurons (Lucas et al., 1992; Marksteiner et al., 1992).

Functional implications

Our results suggest that glutamate, via NMDA and AMPA receptors, contributes to neurokinin signaling in the NST. The NST is a principal site for coordinating the reflex control of autonomic functions. It receives visceral afferent inputs, mainly originating from the vagus nerve and using glutamate as principal neurotransmitter. Interactions between glutamate and neurokinins may be involved in the control of autonomic functions by the NST. First, activation of primary vagal afferents may contribute to substance P/neurokinin A signaling in the NST. Thus, both NMDA and non-NMDA receptors coexist on second-order neurons in the NST and mediate primary visceral afferent transmission in the NST (Aylwin et al., 1997). In addition, some of NST second-order neurons contain substance P (Kawai et al., 1989). Finally, we have shown that both NMDA and AMPA are able to induce neurokinin release in the NST as assessed by NK1 receptor internalization. Second, neurokinins might be part of a mechanism that amplifies glumatergic neurotransmission in NST neurons. Several studies have shown that substance P potentiates NMDA responses in neurons of the spinal dorsal horn (Rusin et al., 1993; Cumberbatch et al., 1995). In addition, substance P via NK1 receptor, post-synaptically potentiates glutamate-induced currents in vagal efferent neurons (Liu et al., 1998).

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