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Neurokinin-1 Receptor Immunoreactive Neuronal Elements in the Superficial Dorsal Horn of the Chicken Spinal Cord: With Special Reference to Their Relationship with the Tachykinin-containing Central Axon Terminals in Synaptic Glomeruli

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Synaptic glomeruli that involve tachykinin-containing primary afferent central terminals are numerous in lamina II of the chicken spinal cord. Therefore, a certain amount of noxious information is likely to be modulated in these structures in chickens. In this study, we used immunohistochemistry with confocal and electron microscopy to investigate whether neurokinin-1 receptor (NK-1R)-expressing neuronal elements are in contact with the central primary afferent terminals in synaptic glomeruli of the chicken spinal cord. We also investigated which neuronal elements (axon terminals, dendrites, cell bodies) and which neurons in the spinal cord possess NK-1R, and are possibly influenced by tachykinin in the glomeruli. By confocal microscopy, NK-1R immunoreactivities were seen in a variety of neuronal cell bodies, their dendrites and smaller fibers of unknown origin. Some of the NK-1R immunoreactive profiles also expressed GABA immunoreactivities. A close association was observed between the NK-1R-immunoreactive neurons and tachykinin-immunoreactive axonal varicosities. By electron microscopy, NK-1R immunoreactivity was seen in cell bodies, conventional dendrites and vesicle-containing dendrites in laminae I and II. Among these elements, dendrites and vesicle-containing dendrites made contact with tachykinin-containing central terminals in the synaptic glomeruli. These results indicate that tachykinin-containing central terminals in the chicken spinal cord can modulate second-order neuronal elements in the synaptic glomeruli.

Key words: electron microscopy, neurokinin-1 receptor, synaptic glomerulus, tachykinin, immunocytochemistry

I. Introduction

The superficial dorsal horn of the spinal cord of the chicken has a unique layered composition, in which lamina I lies most laterally, lamina II lies between lamina I and lamina IV, and lamina III is apparently completely separated from lamina II by lamina IV [4]. Furthermore, laminae III/IV have been shown to exclusively receive myelinated primary afferents that have fast conduction velocity. In contrast, laminae I/II receive both myelinated and unmyelinated primary afferents with slow conduction velocity [37]. In addition, substance P, known to be a neuromodulator released by peripheral noxious stimuli [35, 38], has been detected in axon terminals concentrated in lamina I/II of the dorsal horn of the chicken [11, 26].

There are at least two different interpretations of the relationship between substance P-containing neuronal elements and their targets, which express substance P receptors (neurokinin-1 receptor, NK-1R). Several studies that used immunocytochemical staining of NK-1R have emphasized a discrepancy in the localization of substance P immunoreactivities and NK-1R immunoreactivities [3, 15, 22]. These

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studies suggested that substance P may indirectly influence its targets in the processing of nociceptive sensations, for instance, using volume transmission rather than direct synaptic transmission. However, Naim *et al.* [21] and Todd *et al.* [34] have shown evidence of a close correlation between substance P immunoreactivities and NK-1R immunoreactivities. In addition, McLeod *et al.* [19] and Li *et al.* [13] have reported many NK-1R-immunoreactive profiles apposed by substance P immunoreactive boutons in the rat superficial dorsal horn.

It is easier to compare the distribution of NK-1R with that of substance P in the chicken spinal dorsal horn than in the rat spinal cord because substance P-containing primary afferents are likely to be confined within laminae I/II, which form an area distinct from the other laminae that rarely contain substance P immunoreactivities.

At the electron microscopic level, a certain population of substance P-containing primary afferent axons constitutes synaptic glomeruli as central terminals in the rat superficial dorsal horn [25]. We reported that substance P-immunoreactive primary afferents often formed synaptic glomeruli as central terminals through lamina II of the chicken spinal cord [26]. We also reported that the substance P-immunoreactive central terminals were not only pre-synaptic, but also post-synaptic to glycine-, gamma amino-butyric acid (GABA)- and enkephalin-immunoreactive neuronal elements in the chicken dorsal horn [2, 27]. Therefore, we speculated that nociceptive sensation is likely to be modified through the exchange of information among neuronal elements in the synaptic glomeruli. The previous NK-1R immunohistochemical studies, however, indicated that NK-1Rs are preferentially contained in excitatory neuronal elements in the rat spinal dorsal horn [14]. If this is true for the chicken, the peripheral elements in the synaptic glomeruli that appear to contain inhibitory neurotransmitter [2, 27] may not show NK-1R immunoreactivities. In the above case, substance P in the central axon terminals does not play any role in the activation of peripheral elements, although the peripheral elements and the central axon terminals apparently have a close inter-relationship in the synaptic glomeruli. In other words, there may be a discrepancy between the releasing site and the receiving site of substance P, revealed at an electron microscopic level.

In this study, we aimed to compare the distribution of substance P-containing elements and NK-1R-containing elements in the chicken spinal dorsal horn. We used immunocytochemical double-labeling using a rat monoclonal anti-substance P antibody and a rabbit anti-NK-1R antiserum. We then examined if the NK-1R immunoreactive neurons show GABA immunoreactivities simultaneously using the rabbit anti-NK-1R antiserum and a guinea pig anti-GABA antiserum. We also examined the correlation between the NK-1R and substance P-immunoreactive neuronal elements in the superficial dorsal horn of the chicken spinal cord using confocal microscopy, and then in lamina II at the electron microscopic level.

II. Materials and Methods

All animals used in the present study received humane care in compliance with the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised in 1985).

Confocal microscopy

Two two-month old female chicks (0.70 kg, 0.92 kg) and two adult female chickens (1.76 kg, 1.9 kg) were deeply anesthetized with pentobarbitone and perfused through the left ventricle with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Another two two-month old female chicks (0.85 kg, 0.96 kg) were deeply anaesthetized and perfused through the left ventricle with a fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PB for GABA immunocytochemistry.

Segments of the cervical enlargement removed from the spinal column were cut into 50 μm-thick horizontal and transverse sections on a Microslicer (DSK, Kyoto, Japan). These sections were then treated with 50% ethanol for 30 min to enhance antibody penetration, rinsed extensively in distilled water, and incubated for 3 days at 4°C in the following primary antibodies diluted in phosphate buffered saline (PBS): 1) rat anti-substance P monoclonal antibody (diluted 1:500, Chemicon International, Temecula, CA) and rabbit antiserum to NK-1R (diluted 1:5000, Oncogene Research Products, San Diego, CA, now Calbiochem, La Jolla, CA), 2) rabbit antiserum to NK-1R (1:5000, Oncogene Research Products) and guinea pig antiserum to GABA (1:1000, Chemicon International). After several rinses in PBS, the sections for the NK-1R and tachykinin double-immunolabeling were incubated in biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA), rinsed and incubated in Cy-2-conjugated streptavidin (1:400, Jackson ImmunoResearch, West Grove, PA) for NK-1R immunoreactivities, and goat anti-rat IgG coupled to Texas red (1:200, Vector Laboratories) for substance P (tachykinin) immunoreactivities. Other sections for NK-1R and GABA double-immunolabeling were incubated for 1 day at 4°C in donkey Cy3-labeled anti-rabbit IgG (1:400, Jackson ImmunoResearch) for NK-1R and donkey Cy2-labeled anti-guinea pig IgG (1:400, Jackson ImmunoResearch) for GABA. The sections were rinsed and mounted in anti-fade medium (Shandon, Pittsburgh, PA) and scanned with a Leica TCS4D confocal microscope equipped with a Krypton-Argon laser.

Electron microscopy

Two adult female chickens (1.9 kg, 1.5 kg) were anesthetized and perfused with a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in PB (pH 7.4). Transverse sections (50 μm) were cut with a Microslicer treated with 50% ethanol for 30 min, and rinsed extensively in distilled water. These sections were then incubated for 3 days at 4°C in the anti-NK-1R antiserum, rinsed in PBS, and incubated in biotinylated goat anti-rabbit IgG for 1 hour at room temperature. After further rinsing, sections were incu-

bated for 1 hr with avidin-biotin-peroxidase complexes using an ABC Elite kit (1:50, Vector Laboratories), rinsed, and peroxidase activity was revealed with diaminobenzidine (DAB, 1 mg/ml) in the presence of hydrogen peroxide (0.01%). The sections were then treated with 1% osmium tetroxide for 1 hr, dehydrated and embedded in epoxy resin. Ultra-thin sections were cut and collected on nickel grids. Ultra-thin sections on grids were incubated in rat anti-substance P monoclonal antibody (diluted 1:200, Chemicon International), rinsed, incubated in colloidal gold-conjugated rabbit anti-rat IgG (1:50, Amersham, Arlington Heights, IL), rinsed and counter-stained with solutions of lead citrate and uranyl acetate. The specimens were viewed and typical images were recorded using a Hitachi H-7500 transmission electron microscope equipped with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

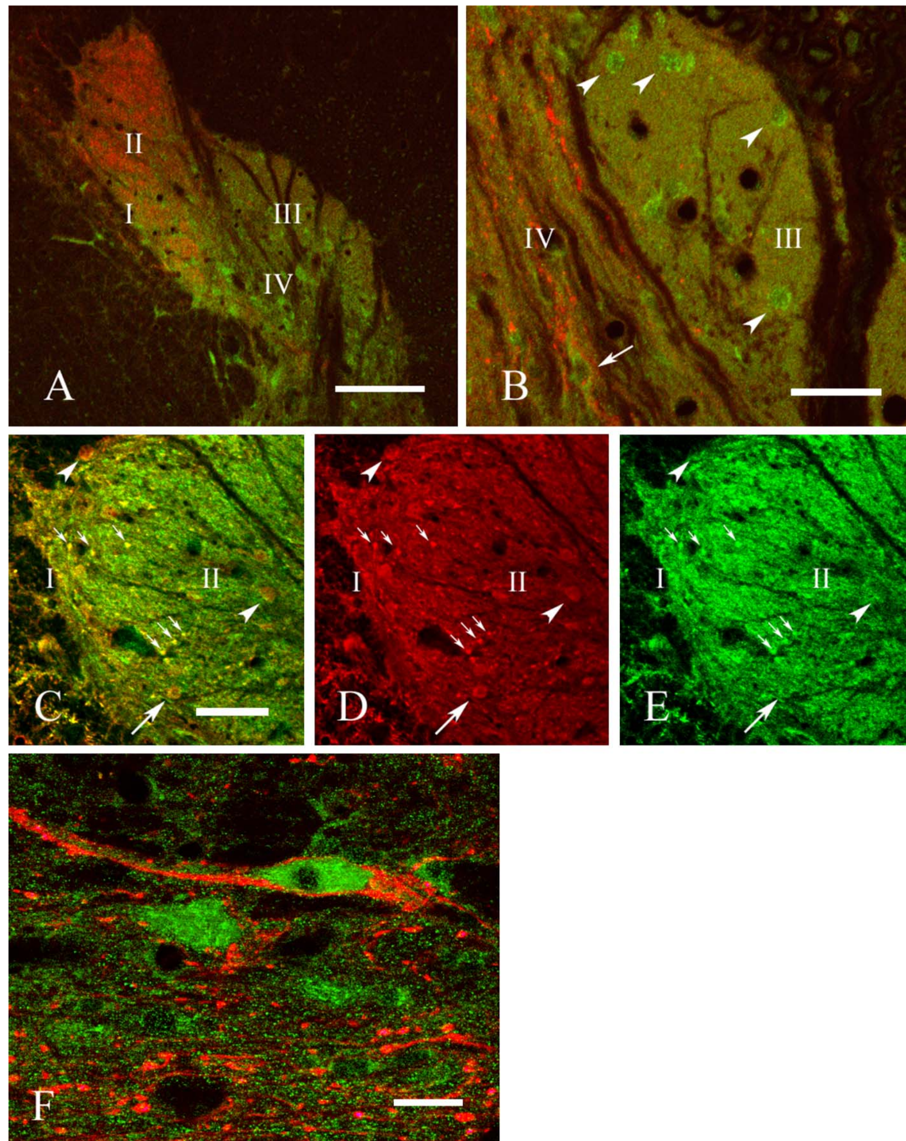

Fig. 1. Confocal images of immunohistochemically double-labeled specimens of the chicken spinal dorsal horn. Each lamina is indicated in Roman numerals. In **A**, tachykinin-(red) and neurokinin-1 receptor (NK-1R)-(green) immunoreactive neuronal elements are shown in low magnification. In **B**, tachykinin (red)-immunolabeled terminals made contacts with an NK-1R (green)-immunolabeled neuronal cell body (arrow) and its dendrites in lamina IV, and several NK-1R-immunolabeled neurons without tachykinin-innervation could be observed in lamina III (arrowheads). In **C–E**, colocalization of GABA (red) immunoreactivity and NK-1R (green) immunoreactivity was examined in the chicken dorsal horn. GABA-immunoreactivities are shown in **D**, those of NK-1R are shown in **E**, and the merged image is shown in **C**. A large arrow indicates a double-labeled neuron, small arrows indicate double-labeled dots, presumably dendrites, and arrowheads indicate GABA-positive- but NK-1R-negative neurons. In **F**, an NK-1R-immunoreactive cell in lamina II, whose dendrites could be traced to a certain extent, receives tachykinin-immunoreactive contacts along the dendrites. A was obtained from five optical sections 1 μm apart from a cervical enlargement transverse section, **B–E** were from three sections and F was from seven sections. Bars=200 μm (**A**), 50 μm (**B, C**), 20 μm (**F**).

Antibody specificity

Antiserum against NK-1R was purchased from Oncogene Research Products. The antiserum was raised in rabbit in response to the peptide KTMTESSSFYSNMLA, which corresponds to a 15-amino-acid section (residues 393–407) of the cytosolic carboxy-terminal region of NK-1R. This antiserum has been shown to recognize a protein band of 80–90 kDa on western blots of lysates from cells transfected with NK-1R; immunostaining could be blocked by the addition of the immunizing peptide [5, 16, 36].

We tested the specificity of the NK-1R antiserum for immunocytochemistry in the chicken spinal cord using antiserum pre-absorbed with the synthetic peptide that is identical to 15-amino-acid section (residues 393–407) of the carboxy-terminal region of NK-1R (AnaSpec, Inc., San Jose, CA.). The specific immunostaining seen in cell bodies, fibers and boutons was abolished after pre-absorption of the antibody with synthetic peptide (200 μg/ml) throughout the chicken spinal cord.

Specificity of the rat monoclonal antibody NC1/34HL antibody against substance P has been demonstrated by Cuello *et al.* [6]. It recognizes the C-terminal part of substance P and does not differentiate substance P from neurokinin A or B [20]. In this study, we refer to immunoreactivities to all three substances as "tachykinin-immunoreactivity". Specific immunostaining in the chicken spinal dorsal horn was abolished after pre-absorption of the antibody with synthetic substance P (200 μg/ml) [26].

The antiserum to GABA was purchased from Chemicon International (Catalogue Number: AB175). The data sheet for the antiserum showed that the polyclonal antibody recognizes GABA and the specific staining with the antibody was blocked by preabsorbing with 100 μM GABA conjugated to glutaraldehyde. Five hundred μM of similar conjugations of glutamic acid, glutamate and taurine failed to block staining.

III. Results

There was no difference in immunoreactivities for NK-1R, tachykinin and GABA between the spinal cord from two month-old chicks and that of adult chickens. We therefore treated the data from two month-old chicks and that of adult chickens equally in this study.

Confocal microscopy

Dense tachykinin-immunoreactivity was seen in laminae I and II (in red in Fig. 1A), as reported previously [26]. Most of the immunoreactive elements were fibers and their swellings as shown in Figure 1F. The other regions of the dorsal horn showed much lower levels of immunoreactivity (Fig. 1A).

NK-1R immunoreactivities were distributed throughout the gray matter of the cervical cord of the chicken. The immunoreactivities were seen on neuronal cell bodies and dendrite-like profiles in the dorsal horn (Fig. 1, in green).

NK-1R-immunoreactive neuronal cell bodies in laminae I–III were fusiform, extending the bipolar dendrites in a rostro-caudal direction, *e.g.* an NK-1R immunoreactive fusiform neuron in lamina II in Figure 1F. In lamina IV, the NK-1R neurons appeared rounder or multipolar and their dendrites extended in multiple directions, occasionally dorso-ventrally (Fig. 1B). NK-1R-immunoreactive neuronal cell bodies were counted and then measured in the minor axis when their entire nuclei were included in the section to avoid double measurement. One hundred and forty three NK-1R-immunoreactive neuronal cell bodies were measured from 42 transverse dorsal horn sections of the chickens' cervical enlargements (Table 1).

NK-1R-immunoreactive neurons in lamina I were a mixed population in size. Measured along the minor axis, the cell size ranged from 6.7 μm to 21.5 μm (mean: 12.5 μm, standard deviation: 4.1, n=25). Lamina II NK-1R-immunoreactive cells were smaller (mean: 9.0 μm, standard deviation: 1.5, n=97). Occasionally, however, we encountered large immunoreactive neuronal cells, up to 29.5 μm, on the border between laminae II and IV. NK-1R-immunoreactive neuronal cell bodies in lamina III ranged in size from 7.5 to 14.1 μm (n=8), and those in lamina IV ranged in size from 9.2 to 27.3 μm (average: 14.9 μm, standard deviation: 4.8, n=13).

Tachykinin-immunoreactive varicosities were often associated with NK-1R-immunoreactive cell bodies and dendrite-like profiles in laminae I, II and IV (Figs. 1B and F). We sometimes observed dense innervation of tachykinin-positive neuronal elements to NK-1R-immunoreactive neuronal cell bodies and dendrites, as shown in Figures 1B and F. In contrast, NK-1R-immunoreactive cell bodies in lamina III rarely received contacts from tachykinin-immunoreactive varicosites (Fig. 1B).

The distribution pattern of GABA immunoreactivities (Fig. 1D) was the identical to our previous results [27]. Immunoreactive neurons were small and seen through laminae I–III of the chicken spinal dorsal horn. Some of the GABA-immunoreactive neurons (10/130) and presumed dendrites (146/684) expressed NK-1R immunoreactivity (Figs. 1C–E) in laminae I, II and in the boundary area between lamina I and the lateral funiculus. The double immunolabeled neurons were between 5.1 and 14.4 μm in the minor axis.

Table 1. *Neurokinin-1 receptor-immunoreactive neuronal cell bodies in the chicken spinal dorsal horn*

	Count	size in the minor axis	
		average±SD	min–max
lamina I	25	12.5±4.1	6.7–21.5
lamina II	97	*9.0±1.5	*5.8–13.1
lamina III	8	11.3±2.5	7.5–14.1
lamina IV	13	14.9±4.8	9.2–27.4
total	143	10.3±3.4	5.8–27.4

* One of the lamina II neurons was extremely large: 29.5 μm in the minor axis. This neuron was excluded from the data shown in this table.

Electron microscopy

We observed NK-1R-immunoreactive neuronal cell bodies in lamina I (*e.g.* Fig. 2A), and smaller NK-1R-immunoreactive neuronal cell bodies, which had thin layers of cytoplasm surrounding their nuclei, in lamina II (Fig. 2B).

In synaptic structures in lamina II, conventional dendrites (indicated as CD in Fig. 2C) and vesicle-containing dendrites (VCD in Figs. 2D, 3B and 3C) showed NK-1R immunoreactivities. Some of the NK-1R-immunoreactive dendrites and vesicle-containing dendrites consisted of synaptic glomeruli, which have been commonly observed in lamina II of the chicken spinal cord [26]. In NK-1R-immunoreactive neuronal elements, DAB reaction products were seen on all membranous structures, and all post-synaptic densities seen were darkened with the reaction products (Fig. 2D).

Using immunohistochemical double-staining, we showed that the central axon terminals (CT) in the synaptic glomeruli, which contained tachykinin immunoreactivities, were directly contacted by NK-1R-immunoreactive peripheral neuronal elements (VCD and CD, Figs. 3A–C). The colloidal gold particles that indicated tachykinin immunoreactivities were observed on dense-cored vesicles in the central terminals. Synapses were confirmed between the tachykinin-immunoreactive central axon terminals and NK-1R-immunoreactive neuronal elements (Figs. 3A–C).

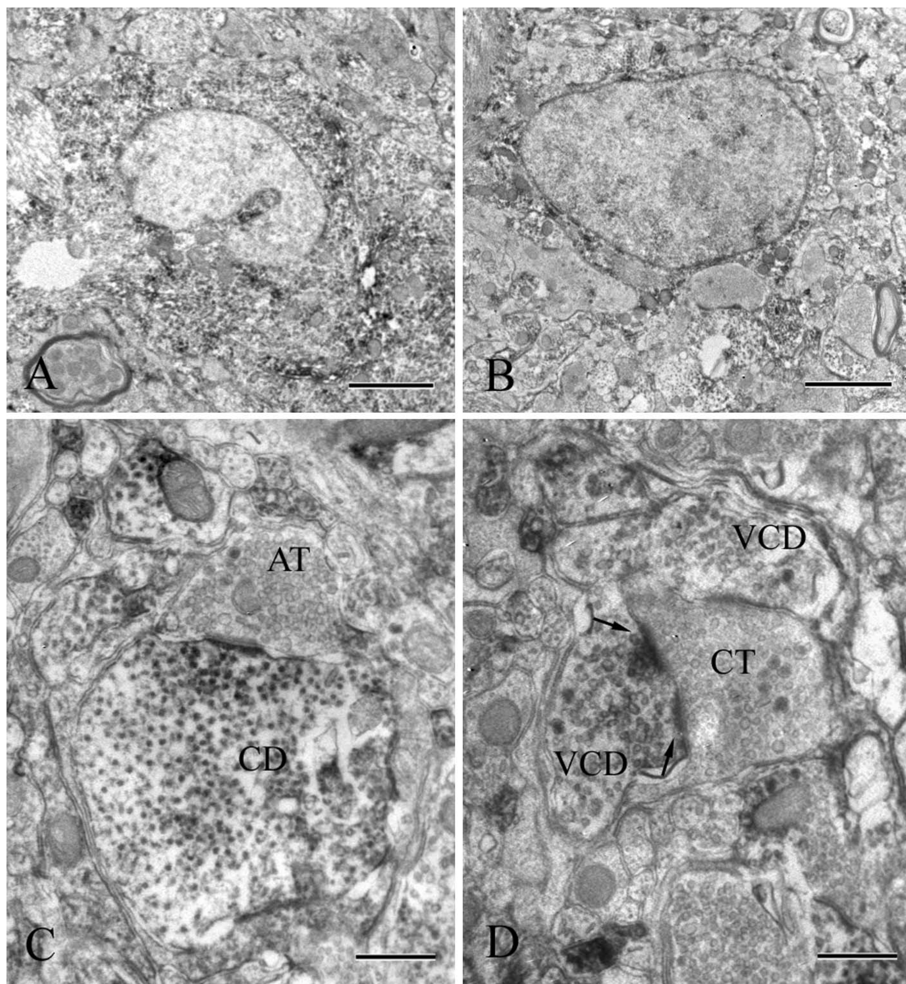

Fig. 2. Electron micrographs of neurokinin-1 receptor (NK-1R)-immunoreactive neuronal elements observed in lamina I (**A**) and II (**B**) of the chicken spinal cervical enlargement. The diaminobenzidine (DAB) reaction product for NK-1R immunoreactivities stained almost every organelle throughout the cytoplasm. As shown in **B**, cell bodies of NK-1R-immunoreactive neurons in lamina II generally consisted of a smaller amount of perikarya. Two subclasses of NK-1R-positive dendrites are shown in **C** and **D**. In **C**, an immunoreactive conventional dendrite (CD) was postsynaptic to an axon terminal or swelling (AT), with numerous clear vesicles and a few dense-cored vesicles. In **D**, two vesicle-containing dendrites (VCDs) made contacts with a non-immunoreactive profile, which appeared to be a central axon terminal (CT) of a primary afferent. One of the vesicle-containing dendrites (VCDs) was clearly stained with postsynaptic densities (arrows). Bars=2 µm (**A**); 0.5 µm (**B, C**).

IV. Discussion

NK-1R-immunoreactive neuronal elements in laminae I and II of the chicken spinal dorsal horn

Neuronal cell bodies

The size of cell bodies of lamina I neurons was diverse, as indicated by the standard deviations and range shown in Table 1. Those cells in lamina I with a larger cell body appear to be projection neurons to the brainstem, as suggested for NK-1R-containing lamina I cells in the rat spinal cord [7, 12, 17, 33, 34]. The cells in lamina II, with thin layers of cytoplasm around their nuclei, and rostro-caudally extended dendritic arborizations, appeared to be local circuit neurons, such as the islet cells identified in other species [9, 10, 24, 30]. In the rat superficial dorsal horn, the inhibitory neurons that contain GABA are reported not to show NK-1R immunoreactivity [14]. We, however, had speculated that some of the GABA neurons of the chicken dorsal horn may express

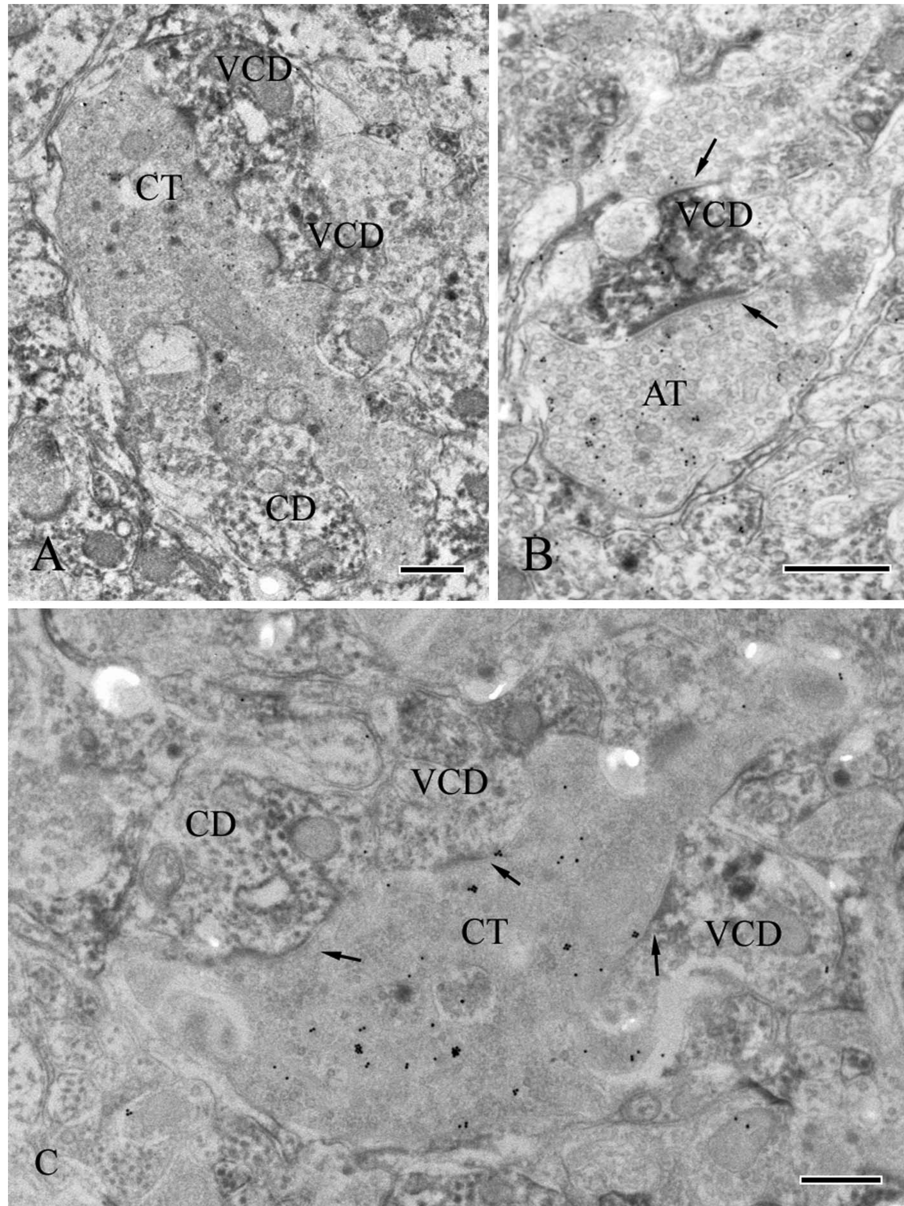

Fig. 3. Double labeling at the electron microscopic level. Neurokinin-1 receptor (NK-1R) immunoreactivity is shown with dark diaminobentizine (DAB) reaction products, and tachykinin immunoreactivity is shown with immuno-gold particles. Several NK-1R-immunoreactive neuronal elements made contacts with a tachykinin-containing central axon terminal (CT) in **A** and **C**. In **A**, two vesicle-containing dendrites (VCDs) made synaptic contacts and a conventional dendrite (CD) apposed a tachykinin-immunoreactive axon terminal (CT). In **B**, an NK-1R-immunoreactive vesicle-containing dendrite (VCD) made two different synapses (arrows) with a tachykinin-immunoreactive axon terminal (AT) and a non-immunoreactive axon terminal. In **C**, two NK-1R-immunoreactive vesicle-containing dendrites (VCDs) and a conventional dendrite (CD) made synaptic contacts (arrows) with a tachykinin-immunoreactive central axon terminal (CT) in a synaptic glomerulus. Bars=0.5 µm (**A**–**C**).

NK-1Rs when we observed that the GABA immunoreactive neuronal elements were in close proximity to and in contact with the tachykinin-immunoreactive central terminals [27]. The fine structural resemblance between GABA-immunoreactive dendrites [27] and NK-1R-immunoreactive dendrites in the present study (Figs. 2C and D) also indicates the possibility. The morphological evidence in our study showed that GABA neurons may be influenced by tachykinin, partially through direct contacts in the chicken dorsal horn. Furthermore, GABA neurons may modulate central terminals soon after they receive tachykinin from the central terminals in the local circuit of the commonly distributed synaptic glomeruli of the chicken spinal dorsal horn.

Dendrites

There were two subclasses of dendrites that showed NK-1R immunoreactivity. One was the subclass of vesicle-containing dendrites that contained pleomorphic clear vesicles with dense-cored vesicles that are likely to come from islet cells in lamina II [31]. The other subclass was conventional dendrites, which seemed to originate from projection neurons from lamina I and/or deeper laminae, or stalked cells of lamina II origin [31]. As we previously reported, a proportion of both subclasses of dendrites in the chicken spinal cord may contain GABA and/or enkephalin [2, 27].

Relationship between NK-1R- and tachykinin-containing neuronal elements in lamina I and lamina II

At the confocal microscopic level, in laminae I and II, NK-1R-immunoreactive neurons often received tachykinin-immunoreactive axon terminals, and some received many of these contacts through their cell bodies and dendrites (Fig. 1C). The close association between tachykinin-immunoreactive axon terminals and NK-1R-immunoreactive dendrites has already been reported in the rat spinal and medullary dorsal horn [13, 19, 21, 28, 34]. We also observed synapses between tachykinin-containing central terminals and NK-1R-immunoreactive neuronal elements in lamina II, at the electron microscopic level. Therefore, we are presenting additional evidence that supports the close spatial relationship between the releasing sites and receptor sites of tachykinin, using the chicken spinal dorsal horn.

Differences in the distribution of NK-1R immunoreactivities between chickens and rats and other mammals

The distribution of NK-1R within the spinal cord and medulla has been investigated immunohistochemically mostly in the rat [3, 5, 12–15, 17, 18, 22, 23, 32, 40]. Lamina II of the adult rat dorsal horn has been reported to display only weak NK-1R-like immunoreactivity, although the greatest concentration of substance P-immunoreactive, *i.e.* tachykinin-immunoreactive, primary afferent axons has been observed in this region [2, 3, 7, 18, 21, 34]. In the present study, a certain number of lamina II neurons expressed NK-1R immunoreactivities as well as lamina I and lamina IV neurons (Fig. 1A). In primate and adult human spinal dorsal horn, the distribution pattern of the NK-1R was reported to be similar to that of rats [8, 39]. The distribution pattern of NK-1R in the chicken spinal cord appears to be unique. Ding *et al.* [8] presented evidence that a certain number of NK-1R neurons exist in lamina II of newborn humans and that these transiently expressed NK-1R-expressing lamina II neurons are the target of the tachykinin-containing primary afferents. Phylogenetically, it is possible that chickens have retained lamina II NK-1R neurons. In addition, in the rat many of the lamina II inhibitory neurons express the AMPA receptor subunit GluR1, which should be the other target of the nociceptive primary afferents [29]. We speculate that neurons that express GluR1 lose the ability to produce NK-1R during development of the rat and other mammals, but retain this productivity in the chicken.

Discrepancy in the distribution of NK-1R- and tachykinin-containing neuronal elements

NK-1R immunoreactivities could be observed in lamina III of the chicken dorsal horn, where tachykinin immunoreactivities have been seen much less frequently (Fig. 1B) and mainly large-diameter primary afferents with faster conduction velocity have been shown to be projected [37]. This would seem to be an example of mismatch between the release sites and receptor sites of tachykinin if NK-1R-immunoreactive elements can only bind to substance P in this area. However, NK-1R binds to other subtypes of neurokinin, such as neurokinin A and B [16]. NK-1R, therefore, may exist in lamina III in order to bind to other subtypes of neurokinin that are released adjacently from neuronal elements in the same lamina. However, this explanation is unlikely, because the monoclonal anti-substance P antibody used in the present studies can bind not only to substance P, but also to neurokinin A and B [20]. Thus, immunoreactivity for tachykinin also implies immunoreactivities for other neurokinins. Another possible interpretation is that NK-1R-positive neurons in lamina III send their dendrites into lamina II to contact tachykinin-releasing elements, as suggested to occur in the rat spinal cord [21]. However, we have so far been unable to show NK-1R-positive lamina III neurons that extend dendrites into lamina II in the chicken spinal cord. Another possible reason for the existence of NK-1R-immunoreactive neurons without tachykinin input in lamina III may be the existence of spare neurons in the case of persistent inflammation. Abbadie *et al.* [1] observed increased NK-1R-immunoreactivity among existing NK-1R-immunoreactive neurons and internalization of NK-1R by the NK-1R-immunoreactive neurons in laminae III–VI in rats with persistent inflammation. These authors indicated that large-diameter primary afferent fibers may start synthesizing tachykinin in a setting with persistent inflammation. NK-1R-immunoreactive neurons in lamina III of chickens may exist to respond to tachykinin that will be provided by large-diameter primary afferent fibers or through volume transmission from other sources in the case of persistent inflammation. We should perform the similar experiment that Abbadie *et al.* [1] reported previously in order to determine the reason for the

NK-1R-immunoreactive neurons without tachykinin input in the dorsal horn of the chicken spinal cord.

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