Substance P and Neurokinin 1 receptor - expression is affected in the ileum of mice with mutation in the W locus

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Abstract

The tachykinin substance P (SP) acts on the gut muscle coat *via* its preferred receptor, neurokinin 1 (NK1r). In the mouse ileum, NK1r-immunoreactivity (NK1r-IR) was detected in neurons, in the interstitial cells of Cajal at the deep muscular plexus (ICC-DMP) and the *myoid cells* of the villi. SP-IR was detected in neurons and varicose nerve fibers, which were especially numerous at the DMP and closely associated with the ICC-DMP. In mice with a mutation in the W locus (c-kit mutant animals), innervation is suggested to be normal although few studies have actually tested this hypothesis. Indeed, studies demonstrating ICC-DMP integrity are lacking and whether SP- and NK1r-IR are normal in these animals has not been investigated. Our aim was to perform an immunohistochemical study on the ileum of a strain of heterozygous mice with a mutation in the W locus, the $W^e/+$ mice, to test this hypothesis. SP-IR nerve fibers were significantly more numerous than in wild type mice; NK1r-IR was clustered on the plasma membrane and also intracytoplasmatic in the neurons, but absent in the ICC-DMP. The richness in SP-IR nerve fibers and the NK1r-IR distribution in the neurons, similar to that of activated cells, might be attempts to compensate for the SP preferred receptor absence at the ICC-DMP. In conclusion, SP content and NK1r expression are noticeably different in c-kit mutants with respect to wild type mice, and probably causing an anomalous tachykininergic control of intestinal motility. Physiological studies on W mutant mice have to take into account that innervation in this animal model is affected by the c-kit mutation.

Keywords: enteric neurons • interstitial cells of Cajal, ICC • immunohistochemistry

Introduction

The tachykinin substance P (SP) is highly represented in the gut muscle coat where it acts *via* its preferred receptor, the neurokinin 1 receptor (NK1r) [1–4]. In the mouse ileum, NK1r-immunoreactivity (IR) has been detected in the submucous and myenteric neurons and in non-neuronal cells, the interstitial cells of Cajal at the deep muscular plexus (ICC-DMP) and the *myoid cells* of the villi [5]. In laboratory mammals, mouse included, SP-IR has been detected in both myenteric and submucous neurons and in varicose nerve fibers distributed throughout the plexuses and musculature [6, 7] and especially numerous in a specific region

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of the circular muscle layer, the DMP, where they form a network closely associated with the ICC-DMP bodies and processes [5, 8]. Generally, SP in the gut is considered as excitatory transmitter that causes muscle contraction acting directly on the smooth muscle cells or indirectly through the activation of enteric neurons [9]; however, it cannot be excluded that some of the SP-containing nerve endings are sensory. Since SP action depends on the specific distribution of the NK1r, circular muscle contraction due to SP might also be mediated through the ICC-DMP [10–13].

Mice with mutation in the W locus (c-kit mutant animals) have an affected intestinal motility that has been considered due to the absence of the ICC population located at the myenteric plexus level, the ICC-MP [14–18]. Conversely, the ICC-DMP are present in these animals, as confirmed by light and transmission electron microscope examination [15–18], and, therefore, it was generally assumed that this ICC type is unaffected in these mutants. However, studies demonstrating a real morpho-functional integrity of these cells are lacking. Moreover, none of the studies on these mice has considered whether SP and NK1r distribution in the ileum is different from normal mice. With this as aim, we presently performed an immunohistochemical study on the ileum of a strain of heterozygous mice with mutation in the W locus, the W^e/+ mice, and compared data obtained with those from normal type. Examination under transmission electron microscope was also performed to confirm that in this mutant strain ICC-DMP are present and ICC-MP absent. Results obtained should help in better understanding whether tachykininergic nerves and NK1r are involved in the impaired intestinal motor activities of the c-kit mutant animals, and also in understanding the role played by the ICC-DMP in intestinal motility of either normal or c-kit mutant mice.

Materials and methods

Animals

Five one-month-old male CD1 mice and 5 mice heterozygous for the allele extreme spotting W^e (W^e/+ mice; Jackson Lab., Bar Harbor, ME, USA) were used. The animals were housed in the cage, had standard dry food and water *ad libitum* and were maintained on a 12-hrs (7:00 a.m. - 7:00 p.m.) light/12-hrs (7:00 p.m. - 7:00 a.m.) dark cycle. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the Committee for Animal Care of the University of Florence (Italy).

Immunohistochemistry

After excision, the ileum obtained from 3 animals for each group was cleaned of digestive material with saline and full-thickness strips 1 cm long were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4, for 6 hrs at 4°C. Tissue specimens were then placed in 30% sucrose in PBS overnight at 4°C and frozen at -80°C. Transverse (cross) sections of 14 μm were cut with a cryostat and collected onto polylysine (0.1% in distilled water) coated slides.

The polyclonal NK1r antibody (a generous gift of Dr. P. Vigna) was raised in rabbit against a synthetic fragment corresponding to the intracellular C-terminal portion of the rat receptor and extensively characterized by radioimmunoassay, immunohistochemistry and Western blotting [19]. The polyclonal SP antibody was raised in rabbit (DBA Italia srl, Milan, Italy). Two series of cryostat sections were made, washed in PBS containing 1.5% normal goat serum and 0.5% Triton X-100 and incubated with the primary antisera. One series was incubated in the presence of the NK1r antibody at the final working dilution of 1:1.000 for 24–48 hrs at 4°C and the second one with the SP antibody at the final working dilution of 1:250, overnight at 4°C in a moist chamber. Negative controls were performed on other four series of sections by omitting the primary antibodies or substituting them with a non-immune rabbit serum in order to check the specificity of the immunostaining. At the end of incubation, sections were rinsed three times in 10-minutes washes in the same buffer as above. After the final wash, the NK1r-immunoreactivity (NK1r-IR) was revealed by incubating the sections in the presence of fluorescein (DTAF)-conjugated pure goat anti-rabbit IgG (H+L) (Jackson Immuno-Research, West Grove, PA, USA) secondary antibody diluted 1:40 for 2 hrs at room temperature. SP-immunoreactivity was revealed by incubating the sections in the presence of rhodamine anti-rabbit IgG F(ab')2 fragment (Boeheringer Mannheim, Germany) secondary antibody diluted 1:20 for 2 hrs at room temperature. The sections
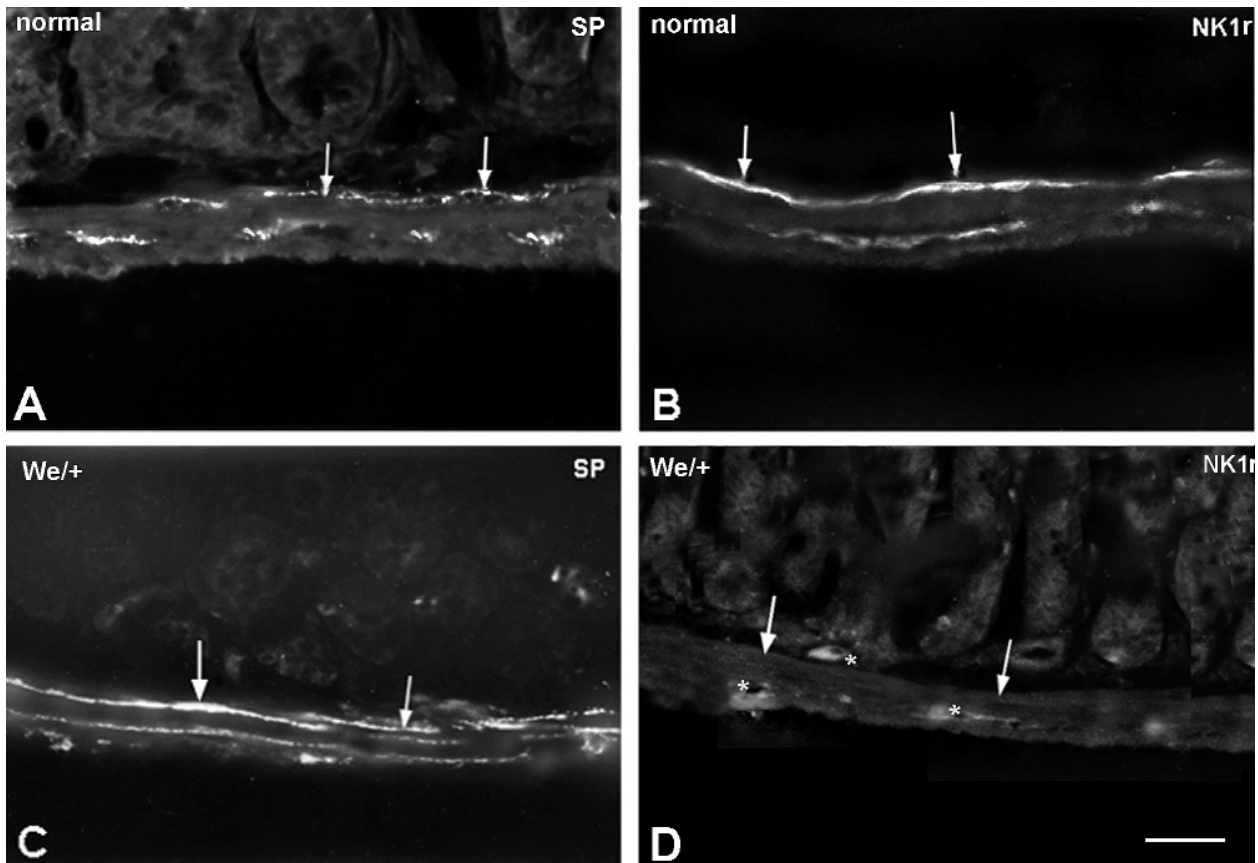
Fig. 1 Mouse ileum, transverse section. **A** and **B**. Normal mice. In A, SP-IR nerve fibers are present within the muscle coat and at the myenteric plexus level. At the deep muscular plexus (*arrows*) the SP-IR nerve varicosities surround SP-negative cells. In B, intensely NK1r-IR spindle-shaped cells (*arrows*) are present at the DMP. NK1r-IR neurons are present within the myenteric ganglia. **C** and **D**. W^e/+ mice. In C, the SP-IR nerve fibers (*arrows*) are particularly numerous at all muscle levels. In D, NK1r-IR cells are completely absent at the deep muscular plexus (*arrows*), while submucous and myenteric neurons (*asterisks*) are NK1r-IR. **A–D**: fluorescence microscope. Calibration bar = 20 μm.

were then mounted in an aqueous medium (Gel Mount, Biomeda corp., Foster City, CA, USA). The immunoreaction products were observed under epifluorescence Zeiss Axioskop microscope and photographed.

Electron microscopy

The tissue, obtained from 2 animals each group, was cut in full-thickness strips and fixed for 4–6 hrs in buffered glutaraldehyde (1.6% in 0.05 M cacodylate buffer, pH 7.4). After four rinses in the cacodylate-buffered solution containing 0.22 M sucrose, the strips were post-fixed for 1–2 hrs with 1% phosphate-buffered OsO_4 (pH 7.4), dehydrated with acetone and embedded in Epon using flat moulds. Semi-thin sections, obtained with a LKB NOVA ultramicrotome, were stained with a solution of

toluidine blue in 0.1 M borate buffer and then observed under a light microscope. Ultra-thin sections of the selected areas were obtained with the LKB NOVA ultramicrotome using a diamond knife and stained with a saturated solution of uranyl acetate in methanol (50:50) per 12 min at 45°C, followed by an aqueous solution of concentrated bismuth subnitrate per 10 min at room temperature. These sections were examined under a JEOL 1010 electron microscope and photographed.

Quantitative analysis

The density of the SP-immunoreactive (SP-IR) nerve fibers in the entire muscle coat (longitudinal and circular muscle layers plus myenteric plexus) was evaluated in transverse sections of normal and W^e/+ mice ileum.

Four photographic fields were taken under a 40x objective from each section (4 sections each animal; 3 animals each group). The area of the muscle wall was 60 μm^2 per each optical field and a total of 48 optical fields per each animal group were considered. Quantitative analysis was done by using Scion Image to measure the mean area ± SEM occupied by the SP-IR nerve fibers in each field tresholding each image for the same value in each group. Statistical analysis was performed by means of Student's t-test. A probability value of less than 0.05 was regarded as significant.

Results

Immunohistochemistry

SP-immunoreactivity (SP-IR)
Normal mice. SP-IR nerve structures were distributed at all levels of the ileal wall. At the enteric ganglia, there were numerous SP-IR nerve fibers all of them rich in varicosities (Fig. 1A). SP-IR neurons were also observed. At the DMP, the SP-IR nerve fibers formed a rich plexus and most of the varicosities surrounded SP-negative spindle-shaped cells (Fig. 1A). Apart from those at the DMP, SP-IR nerve fibers were rarely seen within the circular muscle layer.
W^e/+ mice. The SP-IR nerve structures had a distribution similar to normal animals. However, in the muscle coat, the SP-IR nerve fibers were very numerous at both myenteric and DMP plexuses and also frequent within the circular muscle layer (Fig. 1B).

Neurokinin 1 receptor-immunoreactivity (NK1r-IR)
Normal mice. NK1r-IR was present at both the myenteric and submucous neurons and immunoreactivity was uniformly distributed on the plasma membrane of the perikarya (Fig. 1C, 2A). The ICC-DMP were intensely NK1r-IR and appeared as cells oriented parallel to the major axis of the circular muscle cells and having at the opposite poles two long, main processes (Fig.1C). Immunoreactivity was uniformly distributed along the cell contour at both cell body and processes. As already reported [5], *myoid cells* were also NK1r-IR and labelling was distributed all along their plasma membrane.
W^e/+ mice. NK1r-IR neurons were present in these animals, but IR appeared patchy and was also pre-

sent within the cytoplasm (Fig. 1D, 2B). Conversely, NK1r-IR was never observed at the ICC-DMP (Fig. 1D) and the *myoid cells*.

Transmission electron microscope

Electron microscope examination confirmed that the ICC-DMP were present and the ICC-MP lacking in the W^e/+ mice (Fig. 3A–D), in agreement with similar findings widely reported in literature for other c-kit mutant mice [15, 17]. The *myoid cells* were present both in normal and mutant mice.

Quantitative analysis

The mean ± SEM density of the SP nerve fibers located either intramuscularly or at the myenteric plexus was evaluated and quantitative analysis demonstrated that it was significantly higher in the mutant than in normal mice: 1386 ± 137.2 *vs.* 632.4 ± 34.9 pixels per optical field, P<0.0001.

Discussion

The present findings demonstrate, for the first time, significant differences in SP- and NK1r-expression in the ileum of the W^e/+ respect to normal mice. Surprisingly, possible variations in both SP and NK1r content and distribution were never considered in mice and rats with a mutation in the W locus, although these animals have been widely studied and notwithstanding the importance these variations might have in the altered intestinal motility characteristic of these mutants. Moreover, the lack in NK1r-IR we observed on the ICC-DMP is of particular interest because, contrarily to the ICC-MP, the ICC-DMP were reported to be unaffected in the c-kit mutants.

SP innervation is reported to be abundant in the muscle coat of the ileum of laboratory mammals, mouse included [6, 7, 13], and the present data on normal mice confirm its richness as well as its intramuscular distribution. Interestingly, the SP-IR nerve fibers were found to be significantly higher in density, more than two-fold, in the ileum of W^e/+ with respect to normal mice. The SP preferred receptor, the NK1r, also showed important differences either in cell distri-

Fig. 2 Mouse ileum, myenteric neurons. NK1r-immunoreactivity. **A**. Normal mouse. NK1r-IR is present only on the plasma membrane of perikarya and uniformly distributed. **B**. W^e/+ mouse. NK1r-IR is patchy distributed on the perikaryal contour and also internalized in the cytoplasm. **A** and **B**: fluorescence microscope. Calibration bar = 10 μm.
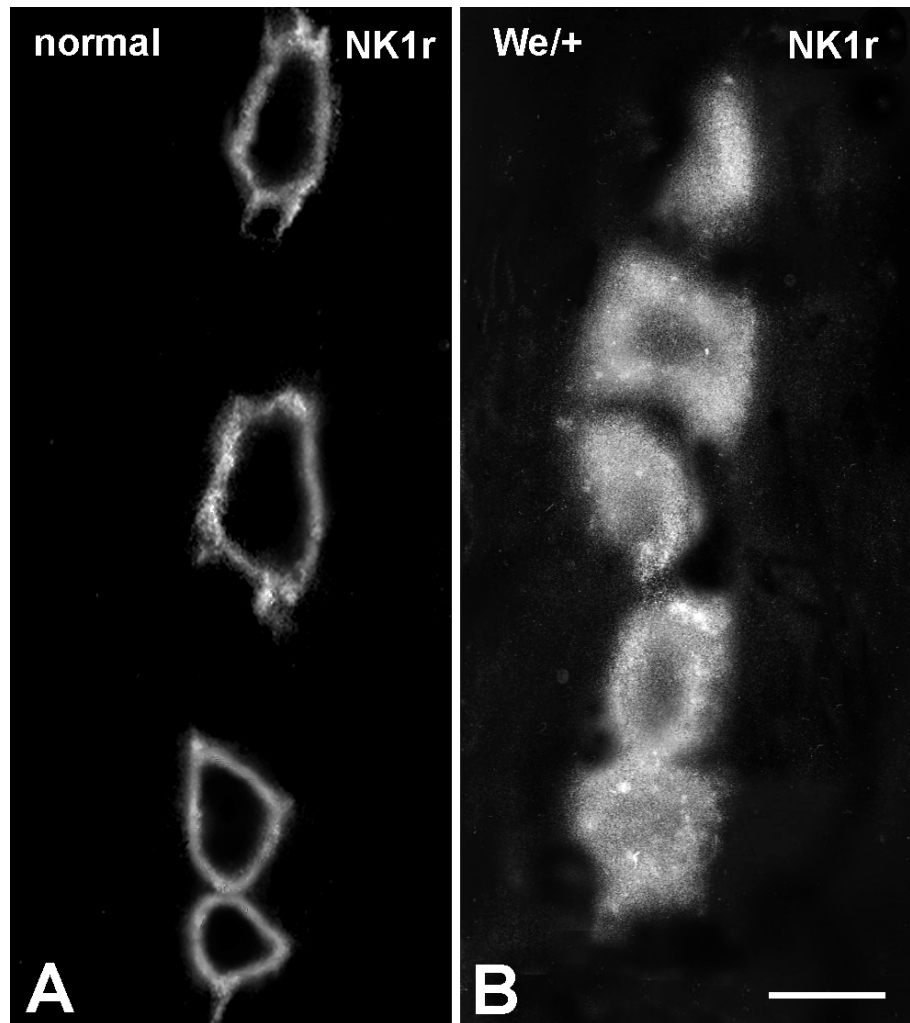
bution or expression. In the neurons, the NK1r–IR was patchy instead of uniformly distributed on the plasma membrane and was present also in the cytoplasm; in the ICC-DMP and *myoid cells,* whose presence in the mutant mice was confirmed by electron microscope examination, the NK1r-IR was not detectable.

The absence of the SP preferred receptor at the ICC-DMP and *myoid cells* is a very intriguing finding. It might be hypothesized that the c-kit mutation causes in some cell types a reduced, undetectable concentration of the NK1r, or a defective transcription and/or translation of this receptor, or the synthesis of a slightly different NK1r not recognized by the antibody presently used. In any case, SP likely does not adequately interact with its preferred receptor at the ICC-DMP and the richness in SP-IR nerve varicosities at the DMP and within the circular muscle layer of these animals can be reasonably interpreted as an attempt to overcome the reduced NK1r availability at the ICC-DMP. The lack of this receptor at

the *myoid cells* is difficult to be understood. These cells have been found to be NK1r-IR only in the mouse and represent a cell type up-to-now few studied although the role they play might be of importance. Indeed, Thuneberg (1999) reported information on these cells collected by old and recent literature and concluded that they form an intravillous network that, similar to the ICC networks in the muscularis, would be spontaneously and rhythmically contracting and, as sensors of stretch, responding to dilation by increased contraction [20]. Conversely, NK1r-IR is present in the neurons of the W^e/+ mice, but its distribution is sign of receptor activation [21] since it is clustered on the plasma membrane and also internalized in the cytoplasm. This peculiar distribution can be related to the extremely high density of SP-IR nerve fibers. Indeed, these fibers presumably release large quantities of SP thus provoking clustering and internalization of its receptor. Briefly, it can be hypothesized that the large amount of the SP-IR nerve
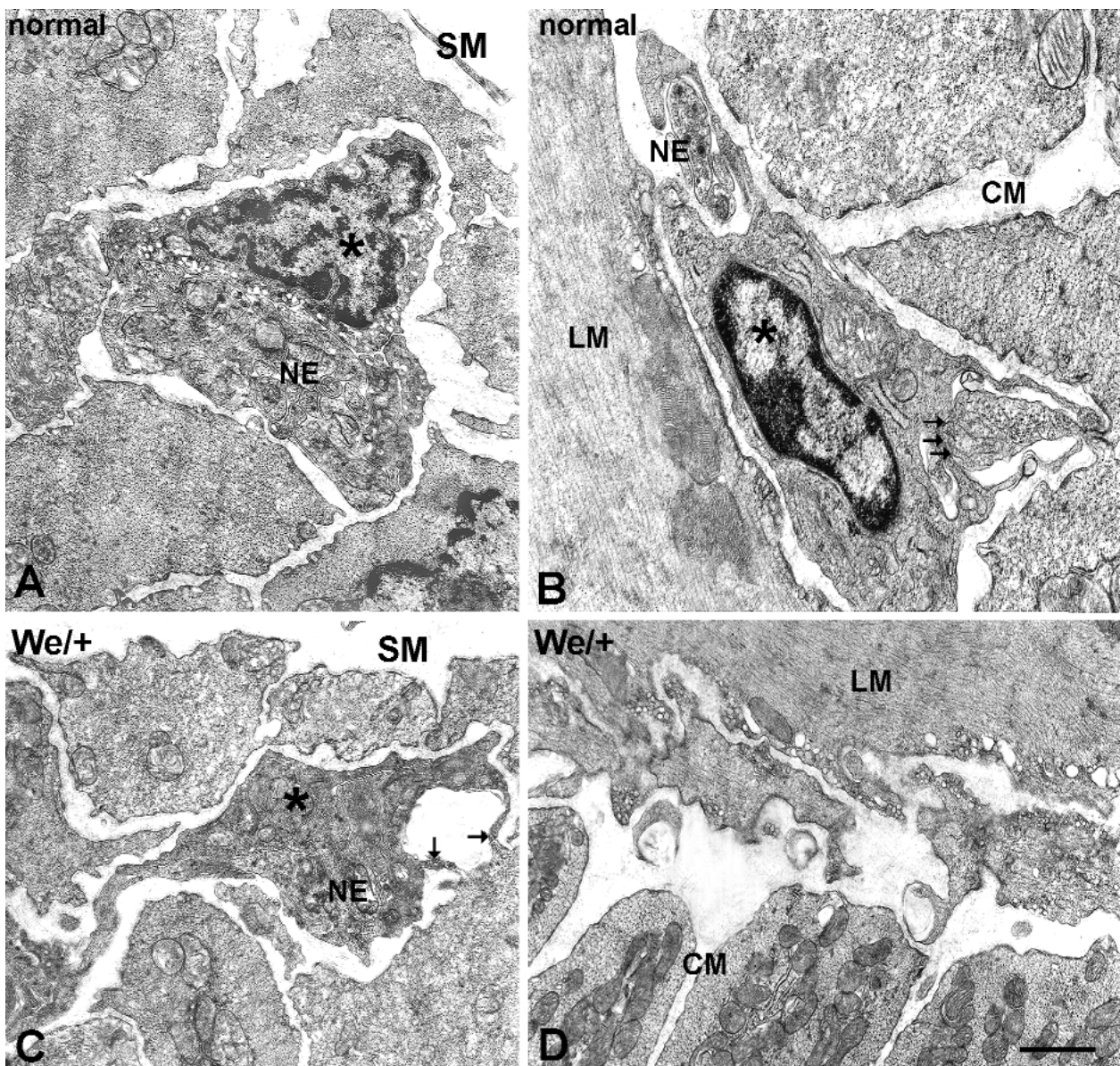
Fig. 3 Mouse ileum, transmission electron microscope. **A**: normal mouse. One ICC-DMP (*asterisk*) close to nerve endings (NE). SM: submucosa. **B**: normal mouse. One ICC-MP (*asterisk*) close to one nerve ending (NE) and in contact (*arrows*) with one smooth muscle cell of the circular muscle layer. CM, circular and LM longitudinal muscle layers. **C**: We/+ mouse. One ICC-DMP (*asterisk*) close to nerve endings (NE) and in contact (*arrows*) with one smooth muscle cell of the circular muscle layer. SM: submucosa. **D**: ICC-MP are not present in between the circular (CM) and longitudinal (LM) muscle layers. Calibration bar: **A** and **C** =1.5 μm; **B** and **D** = 0.8 μm.

fibers in the muscle coat and the NK1r internalization in the neurons might be attempts to warrantee correct muscle contractility and to compensate the excess of SP release. The abundance in SP-IR nerve fibers within the circular muscle should enhance the direct innervation of the smooth muscle cells in the absence or impairment of the tachykininergic inner-

vation of ICC-DMP. No information is available on whether the abnormal intestinal motility found in c-kit mutant animals has an impaired tachykininergic component and on the basis of histochemical data only we cannot make definite conclusions.

Finally, the lack of the NK1r on the ICC-DMP deserves particular attention. This ICC

population was commonly considered unaffected in the c-kit mutant strains, but the present data obtained in the We/+ mice clearly indicate that these cells are functionally impaired. To gain more information on this topic might be of high interest and might also help in understanding the exact role(s) these cells play in gut motility, a role that is still a matter of debate. The ICC-DMP are implicated in inhibitory (nitrergic) [22–25] and excitatory (cholinergic) [25, 26] neurotransmission. It has also been hypothesized that these cells may play a major role in the tachykininergic (excitatory) neurotransmission, since combined studies of immunohistochemistry and pharmacology have demonstrated that the NK1r on ICC is a true receptor [27] and immunohistochemical studies have shown that SP-IR nerve endings are closely apposed to the ICC-DMP [10, 13, 28]. Moreover, although it has not yet been demonstrated, it cannot be excluded that some of the SP-IR nerve varicosities abutting on ICC-DMP are sensory. Recently, these cells have been considered to be directly involved in the stretch reception following distension [29–31] and it has been reported that activation of NK1r on the ileal ICC results in an increase in slow wave frequency and distension-induced peristaltic activity [32]. These data and the present ones supports the presence of the impaired distension observed in the c-kit-mutants [33] and are in favour for an ICC-DMP role in the distension-induced peristaltic activity.

In conclusion, in the ileum of a c-kit mutant mouse, there is presumably an anomalous tachykininergic control of muscle activity, due to lack of NK1r on the ICC-DMP and increased SP nerves within the musculature and plexuses. Hence, c-kit mutations do not exclusively affect ICC but also the enteric nervous system. The study of SP innervation of intestinal muscle may be helped by the discovery of abnormalities in SP innervation of ICC-DMP in c-kit mutant mice.

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