

Expression of vasoactive intestinal polypeptide, substance P and neuropeptide Y in jejunal enteric nerves is altered in rabbits suffering from long term *Trichinella spiralis* infection: an immunohistochemical study

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ABSTRACT: In the early intestinal stage of infection with the nematode *Trichinella spiralis* alterations in gut motility and chemical code of enteric neurons are observed. The present study was designed to characterize the changes in expression pattern of vasoactive intestinal polypeptide (VIP), substance P (SP) and neuropeptide Y (NPY) in enteric nerves of the rabbit jejunum occurring during long-lasting trichinellosis (35 and 42 days). Sections of the jejunum from healthy and *T. spiralis*-infected rabbits were processed for double immunocytochemistry in which antibodies against protein gene product 9.5 were used as a pan-neuronal marker and mixed with antisera raised against VIP, SP or NPY. At 35 and 42 days post infection a marked decrease of VIP-, SP- and NPY-immunoreactive (IR) jejunal myenteric neurons was found, whereas the expression of these neuropeptides in submucous neurons was unchanged. In the myenteric plexus and the jejunal circular muscle of *T. spiralis*-infected rabbits a significant reduction of VIP-IR (but not SP-IR) nerve fibres was noted. In the longitudinal muscle of the jejunum from animals with long-lasting trichinellosis the density of SP-IR nerve terminals was decreased, whereas the number of VIP-containing nerve fibres was unchanged. Long-lasting trichinellosis had no influence on NPY-IR nerve fibres in both circular and longitudinal smooth muscles. The number of NPY-positive (but not VIP- and SP-IR) nerve fibres supplying mucosa and blood vessels was decreased in *T. spiralis*-infected animals. These data indicate that during long-lasting trichinellosis expression of neuropeptides in jejunal enteric neurons is changed. A possible involvement of VIP and SP in persistent intestinal dysmotility and NPY in altered fluid secretion is discussed.

Keywords: neuropeptides; enteric nervous system; neuronal plasticity; trichinellosis; rabbit

The enteric nervous system (ENS) with its unique “brain-like” structure is thought to be a part of the bowel defense line which responds to various mechanical and biological insults such as gut injury, atrophy, inflammation and others. Such adaptive processes aimed at maintenance of the proper intestinal microenvironment are mainly related to the ability of enteric neurons to shift their chemical code, but also involve changes in neuronal excitability and structure (Ekblad and Bauer, 2004). Parasites represent one of the most common gastrointestinal (GI) pathogens; however, depending on the species they may target different parts of

the host GI tract and evoke different symptoms. For example, infection with *Trypanosoma cruzi* (Chagas’ disease) causes neuropathy which reduces the number of enteric neurons and thus leads to the loss of peristalsis in the oesophagus, gastric emptying disorders and disappearance of colonic postprandial motor phenomena (Meneghelli, 1985). In the rat, on day 8 post infection (PI) with *Nippostrongylus brasiliensis* intestinal transit increases whereas on days 10, 12 and 14 no significant changes in relation to healthy animals are observed (Farmer, 1981). In *Trichinella spiralis* parasitized hosts, stimulation of mucosal mast cells to proliferate

erate and release histamine causes local intestinal inflammation (Frieling et al., 1994) and hypermotility (Serna et al., 2006), symptoms which resemble those observed during irritable bowel syndrome. Notably, intense, long term infection of farm animals with trichinellosis (mainly in pigs, cattle and horses) frequently causes weight loss due to reduced productivity, high morbidity and also disqualifies meat intended for human consumption (Eckert, 1996). From this point of view it is highly important to precisely identify parasite-host interactions during infection. Although lines of evidence have indicated that activation of enteric neurons is an important component of an intricate defence process aimed at eliminating *T. spiralis* larvae from the GI tract lumen (Collins et al., 1989; Palmer et al., 1998), the neurochemical mechanisms underlying this action remain to be clarified. It has been discovered that during the first six days of *T. spiralis* infection cholinergic innervation of the rat jejunum undergoes rapid alterations (Collins et al., 1989; Davis et al., 1998) whereas the levels of noradrenaline released by extrinsic nerves projecting to the jejunum fall (Swain et al., 1991). Moreover, in rats intrarectally infected with *T. spiralis* larvae (animal model of ulcerative colitis) several changes in neurochemical properties of enteric neurons were observed at 6 and 14 days post infection (PI; Auli et al., 2008). As the majority of studies focus on the impact of *T. spiralis* larvae on gut function in the early stages of parasite infection, an understanding of the putative changes in innervation pattern of the intestine in long-lasting trichinellosis (when infection has progressed to the muscle phase and adult worms are predominantly to be found in the intestine) is limited.

The aim of the present study was to evaluate whether chronic infection of the rabbit with *T. spiralis* may provoke changes in the chemical content of jejunal enteric neurons and nerve fibres. Therefore, the presence of vasoactive intestinal polypeptide (VIP), substance P (SP) and neuropeptide Y (NPY) in the jejunum was immunohistochemically assessed in normal rabbits and animals in the muscle phase of *T. spiralis* infection (35 and 42 days PI). The choice of the rabbit as an experimental model is justified as this species has been frequently used to study many aspects of trichinellosis and, to the best of our knowledge, there is little data published describing the plasticity of the rabbit's enteric nerves during the muscle phase of nematode infection.

MATERIAL AND METHODS

Animals and tissue sampling

Handling of the animals and experimental procedures were conducted according to guidelines of the local Ethical Committee and were in agreement with Principles of Laboratory Animal Care, NIH publications No. 86-23, revised 1985. Twelve adult female rabbits (weighting approx. 4 kg) were used in the study. Eight rabbits ($n = 8$) were infected by oral administration of rat meat bolus (approx. 1 g) containing *T. spiralis* larvae whereas the animals from the control group ($n = 4$) were fed a placebo. In order to confirm the presence of *T. spiralis* larvae in food samples, trichinelloscopy was conducted prior to experimental infection and the mean concentration of *T. spiralis* larvae was assessed (each portion of meat contained 150–200 larvae per 1 g). After inoculation both the control and experimental animals were kept in cages with free access to food and water.

All rabbits were anesthetized with xylazine (Rometar, Spofa Prague, Czech Republic, 10 mg/kg b.w.) and sacrificed on days 35 and 42 PI/placebo administration. The abdomens were subsequently opened by a midline incision and the jejunum was visualized. From each animal at least six randomly selected samples of the jejunum (each approx. 3 cm long) were dissected out using a pair of sharp scissors. The specimens were opened longitudinally, rinsed with 0.9% sodium chloride and pinned serosa-side up onto a piece of balsa wood. These prepared tissue samples were transferred (three days) into Stefanini solution containing picric acid and paraformaldehyde. The fixative was removed by washing four times (1 per day) in cryoprotective Tyrode solution (4°C). Finally, the specimens were embedded in O.C.T compound and frozen in dry ice. Using a cryostat, serial longitudinal and transverse sections of 10 µm thickness were cut. Every fifth section was placed on a glass slide (SuperFrost® Plus, Mezel, Germany) and stored at –20°C until further immunohistochemical processing.

In order to study the presence of inflammatory infiltrate in the jejunum, every fifteenth section was stained according to the classic hematoxylin and eosin method and viewed under a light microscope (BX-61 Olympus, Nagano, Japan). No inflammatory cells infiltrations were found in normal and *T. spiralis*-infected animals.

For control purposes, in each animal several samples of skeletal muscle were collected *post mortem*

(total weight approx. 50 g) and muscle stage larvae were recovered by artificial digestion with acidified pepsin. In each of experimental animals *T. spiralis* larvae were detected in the sample digest, whereas the musculature of control rabbits contained no muscle stage larvae.

Double immunocytochemistry

Mouse antibodies against protein gene product 9.5 (PGP 9.5; dilution 1 : 80 Abcam, Cambridge, UK; code ab8189) were used as a pan-neuronal marker and mixed with one of the following sera: guinea pig anti-VIP (dilution 1 : 1 200; EuroDiagnostica, Malmo, Sweden; code B-GP 340-1), rat anti-SP (dilution 1 : 100; Biogenesis, Poole, UK; code 8450-0505) or rat anti-NPY (dilution 1 : 500; Biomol, Exeter, UK; code NZ 1115). The sections were placed in a humidified chamber and left to incubate overnight at room temperature (RT) with a mixture of primary antibodies. The sections were then probed with FITC-conjugated anti-mouse goat IgG (dilution 1 : 200; MP Biomedicals, Solon, OH, USA) combined with either Texas Red-conjugated anti-guinea pig goat IgG (dilution 1 : 400; MP Biomedicals) or AMCA-conjugated anti-rat goat IgG (dilution 1 : 100; MP Biomedicals) and left to incubate for one hour in order to visualize primary antibodies. The slides were finally coverslipped with bicarbonate glycerol buffer (pH = 8.6). After each step of immunohistochemical staining three washes with 0.01M phosphate-buffered saline (PBS, pH = 7.3; 10 minutes each) supplemented with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) were carried out.

As a control, the inactivation of primary antibodies with an excess amount of antigen (10–100 µg of synthetic substances per 1 ml of diluted antiserum) was performed. Staining with the pre-absorbed antisera abolished the immunoreaction. As an additional control procedure, the omission of primary antibodies or their replacement with non-immunoreactive sera was carried out. Control stainings yielded no immunoreaction.

Semi-quantification, neuronal counting and statistical analysis

Sections were examined under a spinning disk confocal microscope (BX-DSU Olympus, Nagano,

Japan) equipped with interference filters appropriate for FITC (470–490 nm; MNIBA2), Texas Red (545–580 nm; MWIY2) and AMCA (360–370 nm; MNUA2). All images were captured using a digital color camera (DP-70, Olympus). The density of nerve fibres immunoreactive to the neuropeptides studied was estimated visually according to the following semi-quantitative scale: absent, single, moderate, numerous and very numerous. The ratios of PGP 9.5-positive myenteric/submucous neurons expressing VIP, SP or NPY were expressed as a percentage of the total number of jejunal myenteric/submucous neurons. In each animal, at least six hundred randomly selected enteric jejunal neurons per each staining were assessed (three hundred myenteric, and three hundred submucous neurons). All data were presented as mean ± SEM. The statistical significance of differences between values was analyzed with the one-way analysis of variance test (ANOVA) followed by Bonferroni's post-hoc test at a significance level of $P < 0.05$.

RESULTS

In general, in the rabbit jejunum immunoreactivity to PGP 9.5 was confined both to neuronal cell bodies (myenteric and submucous neurons) as well as to nerve fibres. Neuronal cell bodies stained with antibodies against PGP 9.5 were bright and distinct from the rest of the jejunal tissue. Nerve fibres showed faint immunoreactivity to PGP 9.5 but were distinguishable in comparison to the background. In the rabbit jejunum, PGP 9.5-positive nerve fibres were detected in the smooth muscle layer, myenteric ganglia, the submucous layer (including submucosal blood vessels), submucous ganglia and mucous layer. In normal and infected animals, the expression pattern of PGP 9.5 was comparable and showed no substantial changes.

VIP

In healthy rabbits $2.1 \pm 0.3\%$ of PGP 9.5-IR jejunal myenteric neurons expressed VIP-IR (Figure 1) whereas 35 and 42 days infection with *T. spiralis* this proportion had changed significantly to $0.4 \pm 0.1\%$ ($n = 4$, $P < 0.05$) and $0.8 \pm 0.2\%$ ($n = 4$, $P < 0.05$), respectively. The percentage of VIP-expressing submucous neurons was comparable in non-infected ($5.0 \pm 0.7\%$; $n = 4$, $P < 0.05$) and *T. spiralis*-infected

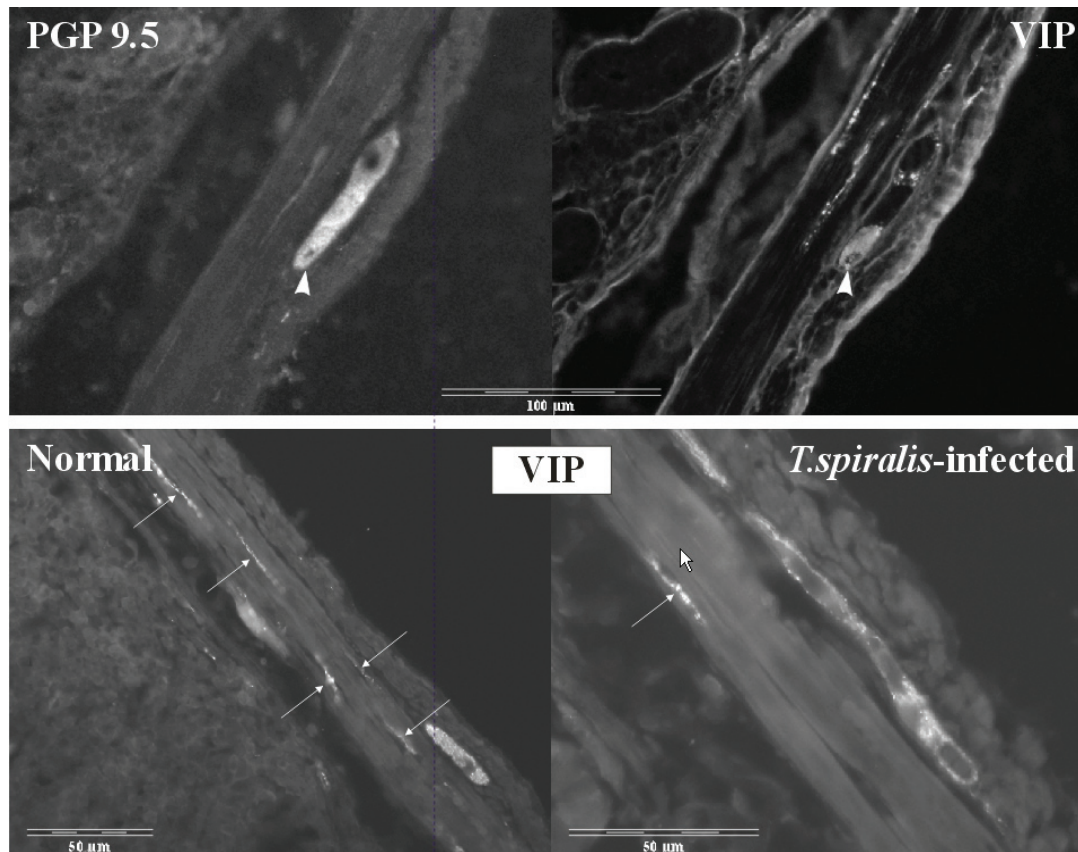


Figure 1. VIP-ergic innervation of the jejunum in normal and *T.spiralis*-infected rabbit (35 days PI). The upper panel illustrates PGP 9.5-IR myenteric neurons from the jejunum of healthy rabbits. Note that only one nerve cell body (arrowhead) is immunoreactive to VIP. In the lower panel the density of VIP-immunoreactive nerve fibres in the circular muscle of normal and *T. spiralis*-infected rabbit jejunum (42 days PI) is presented. Bars represent 100 μ m (upper panel) and 50 μ m (lower panel)

animals ($4.1 \pm 0.3\%$ on 35 days PI and $4.7 \pm 0.5\%$ on 42 days PI). The circular smooth muscle of control animals was innervated at numerous locations with VIP-positive nerve fibres whereas in *T. spiralis*-infected rabbits (35 and 42 days PI) the presence of VIP-IR nerve fibres in the circular muscle was barely detectable (Figure 1). The myenteric plexus of *T. spiralis*-infected rabbits (35 and 42 days PI) was not supplied with VIP-positive nerve fibres in contrast to the situation in healthy animals in which moderately numerous VIP-positive nerve terminals frequently formed basket-like formations around VIP-negative myenteric neurons. The moderate density of VIP-containing nerve fibres found in the longitudinal muscle and lamina propria of the mucosa of normal rabbits was unchanged when compared to *T. spiralis*-infected animals. In the submucous plexus of both control and experimental animals varicose VIP-IR nerve fibres were only occasionally found.

SP

The proportion of SP-IR myenteric neurons in the jejunum of normal rabbits was $8.6 \pm 0.8\%$ ($n = 4$) while the analogous neuronal subpopulation in *T. spiralis*-infected (35 days) animals (Figure 2) was significantly reduced to $1.6 \pm 0.3\%$ ($n = 4$, $P < 0.05$). At 42 days PI the percentage of SP-expressing myenteric neurons was still decreased ($2.1 \pm 0.6\%$; $n = 4$, $P < 0.05$). No SP-positive submucous neurons were found either in normal or *T. spiralis*-infected animals (35 and 42 days PI). The circular muscle of control rabbits was supplied with numerous SP-positive nerve terminals (Figure 2) and a similar density was found in animals with chronic trichinellosis (35 and 42 days PI). SP-containing nerve fibres were present at moderate levels in the longitudinal muscle of healthy rabbits but were found only exceptionally at 35 and 42 days PI (Figure 2). In the myenteric plexus of normal and experimental

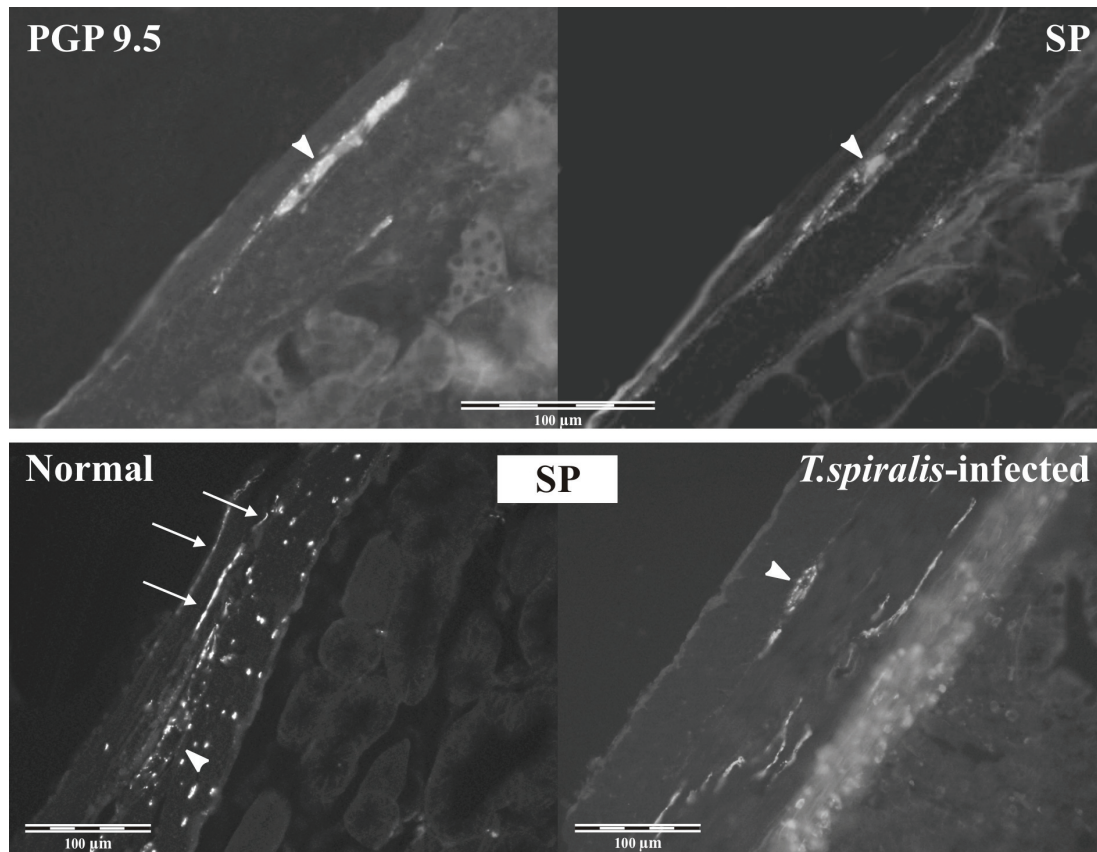


Figure 2. In the upper panel a SP-IR myenteric neuron (arrowhead) from *T. spiralis*-infected (35 days PI) rabbit jejunum is presented. In the lower panel the distribution pattern of SP-positive nerve terminals in the smooth musculature of the jejunum of normal and *T. spiralis*-infected (42 days) rabbits is shown. In both healthy and *T. spiralis*-infected rabbits the circular muscle is supplied with SP-IR nerve fibres whereas the presence of SP in nerve fibres of the longitudinal muscle (arrows) was predominantly found only in control animals. Note that in both uninfected and infected rabbits SP-immunoreactive nerve fibres form basket-like formations around myenteric perikarya (arrowheads). Bars represent 100 μm in both panels

rabbits SP-IR nerve terminals frequently encircled nervous cells. Both in uninfected and *T. spiralis*-infected (35 and 42 days PI) animals SP-IR nerve endings were very rare in the lamina propria of the mucosa.

NPY

In control rabbits immunoreactivity to NPY was found in $6.3 \pm 1.2\%$ of PGP 9.5-positive myenteric neurons, whereas in *T. spiralis*-infected animals a marked reduction of NPY-positive myenteric perikarya (Figure 3) was noted ($0.6 \pm 0.2\%$ at 35 days PI and $1.1 \pm 0.4\%$ on 42 days PI; $n = 4$, $P < 0.05$). In control rabbits and those with long-lasting trichinellosis (35 and 42 days PI) no NPY immunoreactivity was seen in nervous cells of the submucous plexus

(Figure 3) as well as the nerve fibres of the smooth musculature (circular and longitudinal muscles). Moderately numerous submucosal, mainly blood vessel-associated, NPY-positive nerve terminals were observed in healthy animals and this density was drastically reduced in rabbits suffering from trichinellosis for 35 and 42 days (Figure 3). The jejunal lamina propria of normal rabbits contained single NPY-positive nerve fibres which were not seen on 35 and 42 days PI.

DISCUSSION

The present study shows that long term infection with *T. spiralis* (at 35 and 42 days PI) changes the peptidergic innervation pattern of the rabbit jejunum. Previous studies have demonstrated that

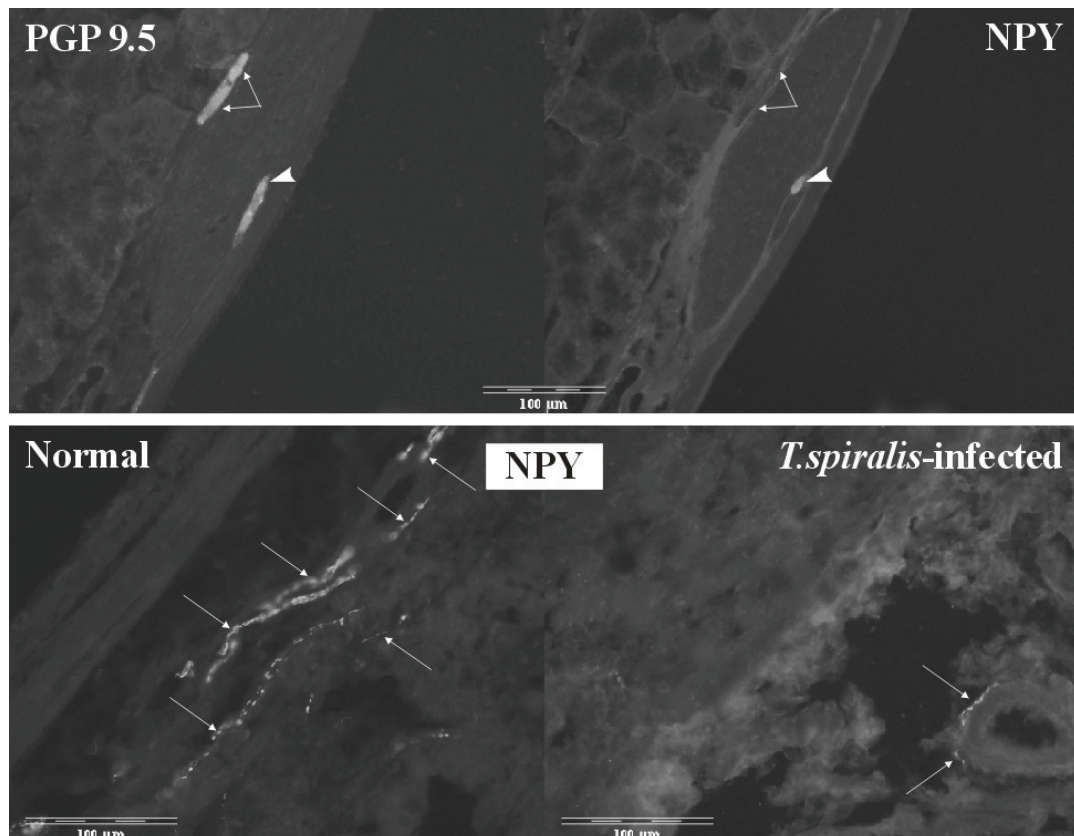


Figure 3. Paired pictures presenting a PGP 9.5-IR/NPY-IR myenteric neuron (arrowhead) found in the jejunum of a *T. spiralis*-infected rabbit (35 days PI) (upper panel). Additionally, in the upper panel two PGP 9.5-positive submucous neurons (arrows) show no presence of NPY. Fluorescence micrographs illustrating the density of NPY-IR nerve fibres (arrows) in the jejunum of control and *T. spiralis*-infected animals (42 days PI) (lower panel). Bars represent 100 μ m (upper and lower)

during the early phase of *T. spiralis* infection enteric nerves undergo alterations in neurotransmitter/neuropeptide content and this phenomenon is thought to be the consequence of *T. spiralis*-evoked intestinal inflammation (Collins et al., 1989; Swain et al., 1991). In the jejunums of *T. spiralis*-infected mice the activity of myeloperoxidase (a marker of acute inflammation) was increased on day 7 PI, remained elevated on days 14 and 21, and normalized after 28 days; the observed changes corresponded to the infiltration of neutrophils and eosinophils (Bercik et al., 2004). It is well known that the intensity of the intestinal inflammatory response to *T. spiralis* infection depends on the number of *T. spiralis* larvae inoculated and that severe inflammation occurs after the oral administration of eight to ten thousand *T. spiralis* larvae (Palmer and Koch, 1995; Palmer et al., 1998). Taken together, the above findings allow us to surmise that in the present study the possibility that inflammatory processes

influenced the chemical content of jejunal enteric nerves was very low. This is due the following facts: to evoke long-lasting *T. spiralis* infections we used a minimal dose of *T. spiralis* larvae; the animals were studied at 35 and 42 days PI and no micromorphological signs of inflammation (inflammatory cells infiltrations) were found in the jejunum.

In the present study, one of the most evident changes observed during long term *T. spiralis* infection was a significant decrease in VIP expression in jejunal myenteric neurons and nerve fibres of the smooth circular muscle. So far, radioimmunoassays have revealed that during the enteric phase of trichinellosis the jejunal concentration of VIP was either unchanged (Palmer and Koch, 1995) or nearly 50% decreased (Palmer and Greenwood, 1993). Moreover, studies involving immunohistochemistry have clearly indicated that in rats with *T. spiralis*-induced colitis no changes in VIP immunoreactivity were to be observed on days 6 and 14 PI (Auli et al.,

2008). Since VIP has been discovered to inhibit the activity of stimulated T cells and the production of pro-inflammatory factors such as tumor necrosis factor α , interleukin-1 β , interleukin-6, interleukin-12 and nitric oxide (Pozo and Delgado, 2004) one could expect that the expression of VIP in the jejunum of *T. spiralis*-infected animals (at least in the early enteric phase) should be up-regulated. Therefore, based on the previous and the present study it is possible that down-regulation of VIP in the jejunum is a long term effect and results from processes other than nematode-evoked inflammation. It is likely that the observed VIP plasticity may be correlated with altered smooth muscle contractility which is observed in *T. spiralis*-infected mice even 42 days PI (Barbara et al., 1997). Since no signs of inflammation are seen 28 days PI, a hypothesis that transient *T. spiralis* infection leads to persistent gut dysfunction has been advanced (Bercik et al., 2004). This concept may be supported by the present finding that changes in VIP expression are predominantly observed in perikarya of the myenteric plexus. VIP-containing myenteric neurons are thought to either project to other myenteric ganglia (Costa and Furness, 1983) or function as inhibitory circular muscle motor neurons (Furness, 2000) which explains simply how VIP down-regulation may lead to disturbances in peristaltic reflex and finally to severe gut dysmotility.

This study indicates that there is a dramatic down-regulation of SP content in jejunal myenteric neurons during long-lasting trichinellosis (35 and 42 days PI). Although, in the inflamed jejunum of parasitized ferrets (Palmer and Greenwood, 1993) and guinea-pigs (Palmer and Koch, 1995) SP concentration was reduced (to 70% and 50% respectively) other studies have shown that in the early phase of rat trichinellosis up-regulation of SP in enteric nerves occurs (Swain et al., 1992, De Giorgio et al., 2001). This latter finding seems to be reasonable because SP is known for its pro-inflammatory properties and its action in the gut is linked with the ability of the neuropeptide to activate and degranulate intestinal mast cells and thus to release pro-inflammatory cytokines (for a review, see Maggi, 1997). Therefore, one could assume that during the post-inflammatory recovery period the number of SP-expressing myenteric neurons should be restored to the basal level. However, at 35 and 42 days post *T. spiralis* infection, to our surprise we found a reduced number of SP-IR myenteric perikarya. In this light the alleged role of endogenously

generated SP during *T. spiralis* infection becomes unclear. It is noteworthy that contradictory observations have also been reported during ulcerative colitis where SP expression in colonic mucosa has variously been found to be decreased (Renzi et al., 1998), not changed (Jonsson et al., 2005) or even increased (Goldin et al., 1989). SP has, however, also been recognized as a neurotransmitter modulating jejunal smooth muscle contractility (Holzer and Holzer-Petsche, 1997). In the small intestine, both the longitudinal and circular muscles are supplied with SP-IR nerve fibres by excitatory motor neurons located in the myenteric plexus (Furness, 2000). This explains why during long-lasting trichinellosis (35 and 42 days PI) the longitudinal muscle was devoid of SP-positive nerve fibres but does not explain the rich SP innervation of the circular muscle. Although this aspect looks to be quite puzzling, it seems that the reduced number of SP-positive nerve fibres projecting into smooth muscles may be one of the reasons for persistent intestinal dysmotility observed after *T. spiralis* infection.

The presented patterns of immunoreactivity to VIP and SP in the jejunum of normal rabbits were similar to previous reports in this species (Keast et al., 1987) albeit the NPY-ergic innervation pattern was slightly different. We were unable to reveal NPY immunoreactivity in both layers of the smooth muscle as well as in the submucous neurons and it is possible that these discrepancies may be due to properties of the primary antibodies used (they were raised in different hosts). So far, no data has been published on whether the content of jejunal NPY is altered during the early phase of trichinellosis. Based on previous reports in different animals, NPY-positive myenteric perikarya (as found herein, markedly down-regulated during long-lasting trichinellosis) may belong to a class of either inhibitory circular muscle motor neurons or mucosa-projecting secretomotor neurons (Furness, 2000). The latter possibility is more likely in the rabbit, as no NPY-IR nerve fibres were noted in the circular muscle and the neuropeptide has been characterized as a potent modulator of epithelial ion chloride secretion (Hubel and Renquist, 1986). Taken together, these findings suggest that during long-lasting trichinellosis the reduced number of intrinsic NPY-IR nerve fibres may result in decreased jejunal fluid secretion. Moreover, it seems that blood flow may be also altered due to the reduced innervation of submucosal blood vessels. It is worth bearing in mind that blood vessel-projecting

NPY-positive nerve fibres are mainly of extrinsic origin (Sundler et al., 1983) which allows us to speculate that during long-lasting trichinellosis a subpopulation of sympathetic neurons, originally NPY-positive, may also undergo several changes in neurochemical phenotype. It must also be considered that the observed lower number of NPY- VIP-, SP-IR and/or neurons and nerve fibres may be due to a higher release of the particular neuropeptide which “empties” the enteric nerves. Such a situation is observed during the early stage of infection, when one of the gut’s priorities is to get rid of nematodes from the gut lumen; one of the ways to achieve this goal is to increase secretion of neuropeptides from the enteric nerves.

In summary, in the present preliminary study we immunohistochemically characterized changes in the peptidergic innervation pattern of the rabbit jejunum which occur during long term infection with *T. spiralis*. We found that long-lasting trichinellosis (35 and 42 days PI) predominantly affected various functional subtypes of myenteric neurons. Whether or not alterations in neuropeptide expression underlie persistent gut dysfunction after *T. spiralis* infection should be the subject of future studies.

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