

## Expression of corticotropin releasing factor (CRF) in the enteric nervous system of the jejunum of sheep

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**ABSTRACT:** Corticotropin releasing factor (CRF), a 41-amino acid neuropeptide widely distributed in the mammalian central nervous system, has been shown to influence several gastrointestinal functions. Recent studies show that CRF released locally from enteric nerves may also underlie alterations in gut function. In this study, immunohistochemistry was applied to demonstrate the presence of CRF in the jejunum of sheep. Using double immunohistochemical staining the co-localization of CRF with vasoactive intestinal peptide (VIP), galanin, tyrosine hydroxylase (TH), neuropeptide Y (NPY) and substance P (SP) was evaluated. The presence of CRF was detected in myenteric neurons ( $3.6 \pm 0.9\%$ ) as well as in submucous neurons ( $10.5 \pm 1.2\%$ ). In the ovine jejunum different numbers of CRF-expressing nerve fibres were detected in myenteric ganglia, submucous ganglia, circular smooth muscle layer, lamina muscularis mucosae and between mucosal glands. None of the CRF-positive enteric neurons and CRF-positive nerve fibres exhibited the presence of TH. CRF-immunoreactive (IR) myenteric neurons widely co-expressed VIP and/or NPY. A minor population of CRF-IR myenteric neurons additionally co-stored SP. Galanin was not present in CRF-IR myenteric neurons. The presence of VIP was observed in the vast majority of CRF-positive submucous neurons. Moderate numbers of CRF-IR submucous neurons co-expressing galanin or NPY were also found. The presence of SP in CRF-positive submucous neurons was noted only incidentally. In the circular smooth muscle layer CRF-IR/VIP-IR, CRF-IR/NPY-IR as well as CRF-IR/SP-IR nerve fibres were present. In the mucosal layer of the ovine jejunum CRF-IR nerve fibres co-stored additionally VIP, galanin, NPY or SP. This present study provides for the first time evidence that CRF present in different subclasses of enteric neurons may influence certain activities of the ovine jejunum. Co-localization studies indicate that VIP, galanin, SP and NPY functionally co-operate with CRF in the jejunum of the sheep.

**Keywords:** enteric nervous system; myenteric neurons; submucous neurons; corticotropin releasing factor; jejunum; small intestine; sheep

Corticotropin releasing factor (CRF) is a 41-amino acid neuropeptide originally isolated from the ovine hypothalamus (Vale et al., 1981). Molecular studies in rodents revealed that CRF is derived from a precursor protein (prepro-CRF) consisting of 187-amino acid residues (Jingami et al., 1985). In addition to CRF other peptides like urocortin I, II and III (UcnI, UcnII and UcnIII), sauvagine and urotensin I also belong to the CRF family. Several pharmacological studies have indicated that CRF exerts its biological action via seven transmembrane G-protein coupled receptor subtypes. To date, three distinct mammalian CRF receptors (CRF1, CRF2a and CRF2b) have been identified and cloned (Grigoriadis et al., 1996). Over the last decades the distribution pattern of CRF-expressing

structures has been well characterized in discrete regions of the central nervous system (CNS). In the CNS, a high concentration of CRF-expressing neurons was observed in the paraventricular nucleus of the hypothalamus (Sawchenko et al., 1984), central nucleus of the amygdala (Moga and Gray, 1985) and parabrachial nucleus (Pammer et al., 1988). A series of experimental studies clearly demonstrated that CRF is a key factor involved in brain function, including coordination of autonomic and behavioural responses to stress, stress-related neuropsychiatric disorders and drug addiction (for a review see Sarnyai et al., 2001). Moreover, it has been documented that CRF may affect gut function. Central administration of CRF evoked inhibition of gastric motility, emptying, and acid secretion as well

as stimulation of colonic propulsion and these effects were mediated through both CRF1 and CRF2 receptors (Druge et al., 1989; Martinez et al., 1998; Martinez and Tache, 2001). Additionally, CRF when administrated peripherally also inhibited gastric emptying (Martinez et al., 1999) as well as increasing duodenal (Mayer et al., 1992), ileal (la Fleur et al., 2005) and colonic motility (Tsukamoto et al., 2006). Recently it has also been shown that CRF has pro-inflammatory properties in the mouse ileum (Wlk et al., 2001). In view of the previous findings, it is apparent that the enteric nervous system (ENS) is of particular significance in CRF signalling pathways affecting the gut. This is due to the fact that both CRF and CRF-receptors are present in enteric neurons (Chatzaki et al., 2004; Porcher et al., 2005; Liu et al., 2006; Sand et al., 2011) and peripheral infusion of CRF activates certain populations of CRF receptor-bearing enteric neurons (Yuan et al., 2007).

While substantial efforts have been made to characterise the occurrence of CRF in the neuronal elements of the rodent gastrointestinal tract (GIT), CRF-ergic gut innervation in higher mammals has attracted relatively less attention. Therefore in the present study, the presence of CRF in the ovine jejunum was immunohistochemically examined. Additionally, by means of double immunocytochemistry the co-expression patterns of CRF with vasoactive intestinal peptide (VIP), galanin, tyrosine hydroxylase (TH), neuropeptide Y (NPY) and substance P (SP) were examined, in order to better understand whether these substances may be functionally involved in CRF-dependent mechanisms. We have chosen the sheep as an experimental model because ruminants have a unique GIT anatomy and physiology and, to our best knowledge, no studies describing the presence of CRF in the ENS have been reported in this species.

## MATERIAL AND METHODS

### Animals

Five young male sheep weighing approx. 10 kg were used. The experiments were approved by the local Ethical Committee and all experimental procedures were conducted in accordance with the Principles of Laboratory Animal Care, NIH publications No. 86-23, revised 1985. The animals were anaesthetized with xylazine (Rometar, Spofa Prague, Czech Republic, 0.4 mg/kg b.w.) and sac-

rificed with an overdose of sodium pentobarbital (Pentobarbitalnatrium, Apoteket, Sweden; 35 mg/kg b.w.). A midline incision of the abdomen was made and the jejunum was taken out and immediately rinsed in cold (4 °C) 0.9% NaCl solution.

### Tissue preparation

Specimens from the jejunum (approx. 3 cm long;  $n = 5$ ) were opened along the mesenteric border and pinned serosa side up on a piece of balsa wood. Jejunal samples were then immersed for 48 h with fixative Stefanini's solution (containing paraformaldehyde and saturated picric acid) at +4 °C. The fixative was removed and the tissue samples were cryoprotected by rinsing in cold 10% sucrose-containing Tyrode's solution (one change per day). Finally, the material was mounted on a wooden block, embedded in O.C.T. compound and left to freeze in dry ice. Transversal and longitudinal sections of 10 µm thickness were made using a cryostat. Every fifth section was placed on glass slides (SuperFrost Plus, Menzel GmbH & CoKG, Germany) and processed for immunohistochemistry.

### Immunohistochemistry

To permeabilize the tissue, preparations were rehydrated in 0.01M phosphate-buffered saline (PBS; pH = 7.3) containing 0.25% bovine serum albumin and 0.25% Triton X-100 (Sigma-Aldrich, MO, USA) at room temperature (RT; 3 × 15 min). Thereafter, the sections were placed in a humidified chamber and incubated overnight at RT with a mixture of primary antibodies raised in different species. In the current studies, rabbit antibodies raised against CRF (1 : 80; Sigma-Aldrich, Germany, C5348) were combined with one of the following antisera: mouse anti-Hu C/D (1 : 400; Molecular Probes, OR, USA; A-21271), mouse anti-vasoactive intestinal peptide (VIP; 1 : 100; Biogenesis, UK, code 9535-0504), mouse anti tyrosine hydroxylase (TH; 1 : 100, Sigma-Aldrich, Germany, T2928), rat anti-substance P (SP; 1 : 200, AbDSerotec, UK, code 8450-0505), rat anti-neuropeptide Y (NPY; 1 : 700, Biomol, UK, code NZ1115) and guinea-pig anti-galanin (1 : 300; Peninsula, USA, code T-5027). Excess primary antibodies were removed by washing the samples in PBS (3 × 15 min). To visualize bound primary antisera

a mixture of appropriate secondary antibodies was used. Cryostat sections were incubated (RT, 1 h) with Texas Red-conjugated anti-rabbit goat IgG (dilution 1 : 400; MP Biomedicals, OH, USA), combined with either FITC-conjugated anti-rat goat IgG (dilution 1 : 400; MP Biomedicals), FITC-conjugated anti-mouse goat IgG (dilution 1 : 400; MP Biomedicals), or FITC-conjugated anti-guinea-pig goat IgG (dilution 1 : 400; MP Biomedicals). After the final wash in PBS (3 x 15 min) the slides were mounted in phosphate-buffered glycerol (pH = 8.2).

As a control, the specificity of positive staining was tested using a preabsorption study (10–100 µg of corresponding blocking substance was added to 1 ml of diluted antiserum). Staining with the pre-absorbed antisera abolished the immunoreaction. Incubation of cryostat sections in solution lacking primary antibodies also served as a negative control. The slides were viewed with a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with interference filters appropriate for Texas Red (545–580 nm; MWIY2) and FITC (470–490 nm; MNIBA2). All images were acquired using a digital colour camera (DP-70, Olympus) and Cell<sup>^</sup>M software (Olympus).

### Semi-quantification and statistical analysis

For statistical assessment, the numbers of AQP1-immunoreactive myenteric as well as submucous neurons were expressed as a percentage of the total number of Hu C/D-positive perikarya analyzed. In each animal, at least 300 myenteric and 300 submucous neurons were studied. The expression of CRF in enteric nerve fibres was estimated visually according to the following semi-quantitative scale: absent, single, moderate, numerous and very numerous. The same scale was also applied in co-localisation studies (co-expression of CRF with VIP, galanin, SP, NPY or TH). Numerical values are presented as means ± SEM. Differences between means were analyzed by analysis of variance followed by Bonferroni's *post hoc* test and  $P < 0.05$  were considered significant.

## RESULTS

In general, the expression of CRF was detected both in enteric neurons as well as enteric nerve fibres of the ovine jejunum. In myenteric ganglia very numerous varicose CRF-positive nerve fibres

were found between Hu C/D-positive myenteric perikarya (Figure 1A). CRF-expressing nerve fibres ran in close vicinity to myenteric neurons and frequently created “ring-like” formations. Only  $3.6 \pm 0.9\%$  ( $n = 5$ ) of myenteric neurons were immunoreactive (IR) to CRF (Figure 1A). Rich varicose nerve bundles showing immunoreactivity to CRF were also detected in submucous ganglia (Figure 1B). In submucous ganglia, CRF-expressing nerve terminals regularly surrounded submucous neurons. Of Hu C/D-labeled submucous neurons a mean of  $10.5 \pm 1.2\%$  ( $n = 5$ ) were immunopositive to CRF (Figure 1B). The subpopulation of CRF-expressing submucous neurons was statistically higher than the subpopulation of CRF-IR myenteric neurons (ANOVA;  $P < 0.05$ ). Moderate numbers of CRF-expressing nerve fibres were detected in the circular smooth muscle layer (Figure 1A), whereas in the longitudinal smooth muscle layer no expression of CRF was detected. Moderate numbers of CRF-positive nerve fibres were additionally noted in the lamina muscularis mucosae (Figure 1C). The presence of CRF-expressing nerve terminals was regularly observed between mucosal glands (Figure 1D). In the submucous layer no expression of CRF was found (including blood vessels). No endocrine cells of the jejunum showed immunoreactivity to CRF.

The presence of VIP was observed in the vast majority (approx. 95%) of CRF-positive myenteric neurons as well as in approx. 90% of CRF-positive submucous neurons (Figure 2A). A large number of CRF-expressing nerve fibres found in the enteric ganglia (both myenteric and submucous), circular smooth muscle layer and around mucosal glands showed co-incidence with VIP (Figure 2B). Only single CRF-positive nerve fibres lacking VIP were noted in the circular smooth muscle layer and between the mucosal glands. In the ovine jejunum, VIP-positive nerve terminals lacking CRF were frequently detected in the longitudinal smooth muscle layer, submucous layer as well as between mucosal glands (Figure 2B).

In the myenteric plexus, neither CRF-positive ganglionic neurons nor CRF-positive nerve fibres showed additionally the presence of galanin (Figure 2C). Also CRF-expressing nerve terminals located in the circular smooth muscle layer were galanin-negative. The expression of galanin was noted in approx. 20% of CRF-IR submucous neurons. The majority of CRF-expressing nerve fibres located in the lamina muscularis mucosae were additionally immunopositive to galanin. The co-localization of CRF and galanin was also visualized in numer-

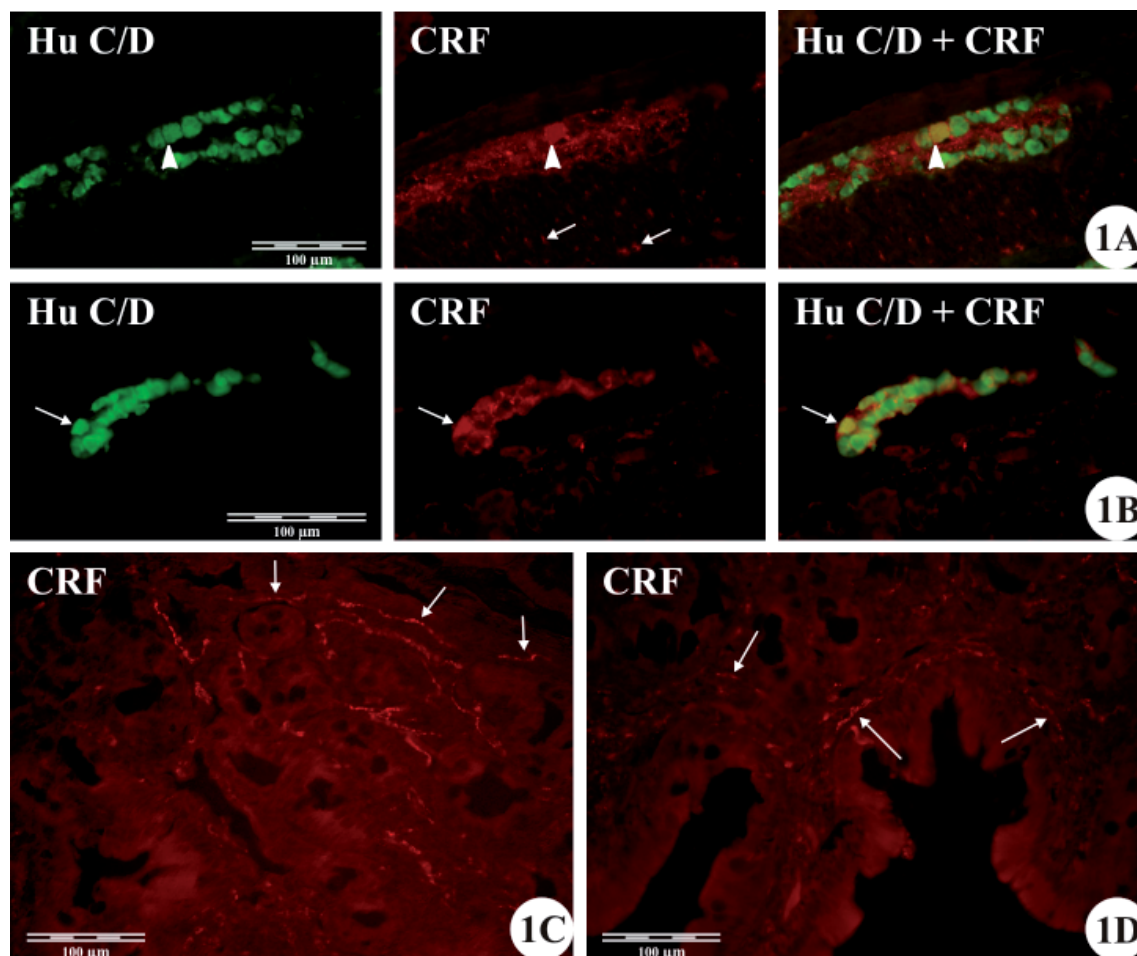


Figure 1. Fluorescence micrographs showing the expression of CRF in the ENS of the ovine jejunum. In (1A) and (1B) antibodies raised against Hu C/D were used as pan-neuronal markers which allowed the detection of CRF-expressing myenteric as well as submucous neurons (arrowhead in 1A and arrow in 1B). In (1A) punctate CRF-positive nerve fibres (arrows) supplying the circular smooth muscle layer are also visible. Note that in both myenteric and submucous ganglia rich networks of CRF-IR nerve terminals running between Hu C/D-positive enteric nervous cells are present (multicolour pictures in 1A and 1B). The expression of CRF in nerve fibres innervating the lamina muscularis mucosa (arrows in 1C) as well as mucosal glands (arrows in 1D) is presented

ous nerve fibres located between mucosal glands (Figure 2D). Moderate numbers of CRF-positive nerve fibres lacking galanin were also observed in the mucous layer of the ovine jejunum (Figure 2D).

The vast majority (approx. 95%) of examined CRF-positive myenteric neurons additionally showed the presence of NPY, whereas approx. 40% of CRF-IR submucous neurons were NPY-positive. Both in myenteric as well as submucous ganglia numerous CRF-IR/NPY-IR nerve fibres frequently encircled enteric neurons (myenteric and submucous). In large numbers of CRF-positive nerve fibres supplying the circular smooth muscle layer the presence of NPY was observed (Figure 3A). The co-incidence of CRF and NPY was additionally detected in nerve fibres

located in the lamina muscularis mucosae as well as between mucosal glands (Figure 3B). In the mucosal layer moderate numbers of CRF-positive nerve terminals lacking NPY were also found (Figure 3B).

Immunoreactivity to SP was detected in approx. 10% of CRF-positive myenteric neurons whereas CRF-IR/SP-IR submucous neurons were rare (approx. 5%; Figure 3C). Moderate numbers of CRF-IR/SP-IR nerve fibres were noted in both myenteric and submucous ganglia. The co-incidence of CRF and SP was also visualized in numerous nerve fibres supplying the circular smooth muscle layer, the lamina muscularis mucosae as well as between mucosal glands (Figure 3D). In the mucosal layer SP-IR nerve terminals lacking CRF were also frequently observed (Figure 3D).



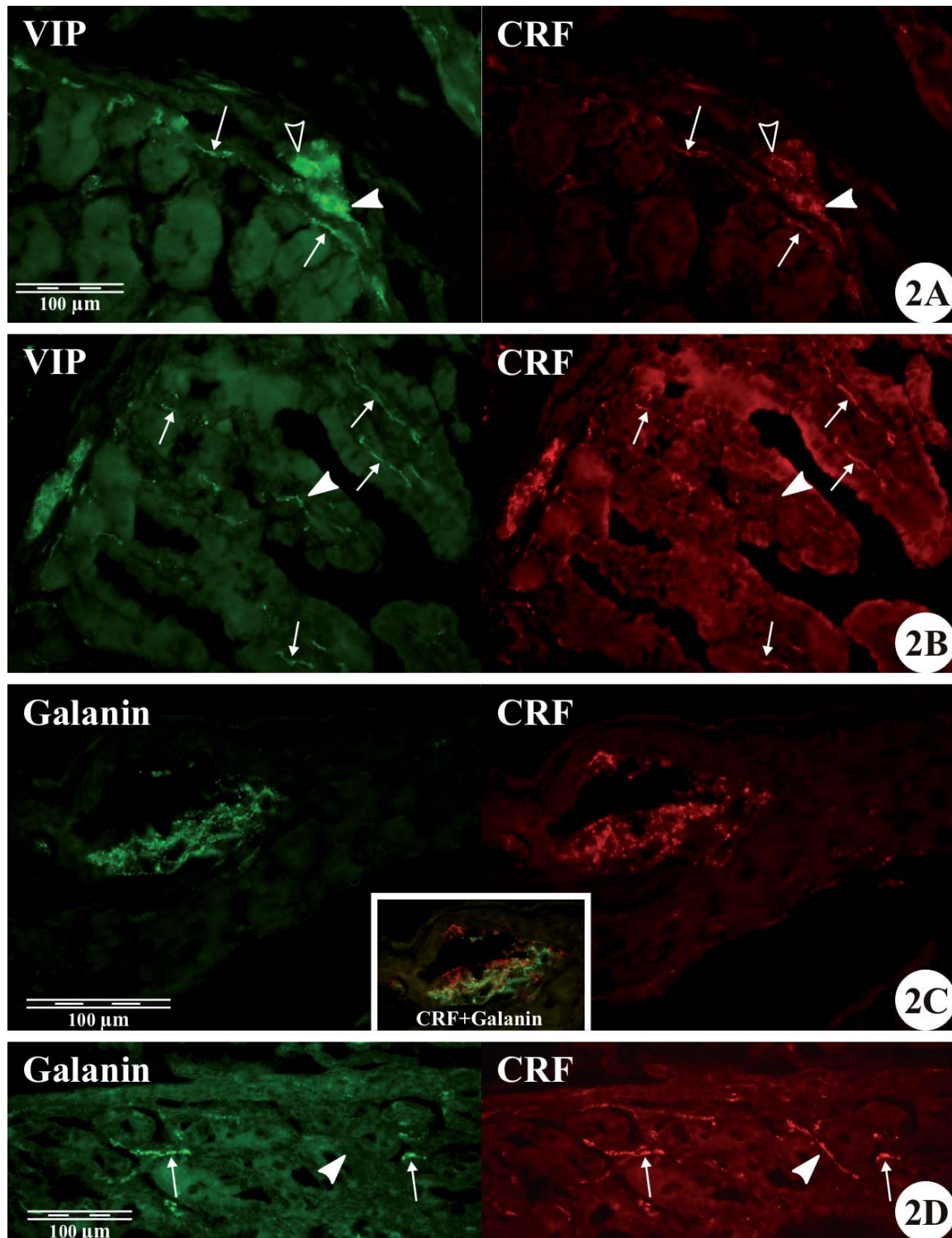


Figure 2. Paired fluorescence micrographs showing the co-expression of CRF with VIP or galanin in the ovine jejunum. Figure (2A) presents CRF-positive/VIP-positive submucous neurons (arrowhead) as well as CRF-positive/VIP-positive nerve fibres of the lamina muscularis mucosae (arrows). In (2A) a SP-IR submucous neuron lacking CRF is also marked with a hollow arrow. In (2B) the co-expression of CRF and VIP is seen in nerve fibres supplying the mucosal glands (arrows). In (2B) note also VIP-positive/CRF-negative nerve terminals (arrowhead). (2C) Galanin and CRF do not co-localise in nerve fibres of myenteric ganglia (which is also visualized in the multicolour combined picture). (2D) CRF-IR/galanin-IR (arrows) as well as CRF-positive/galanin-negative nerve fibres (arrowhead) were distributed between the mucosal glands

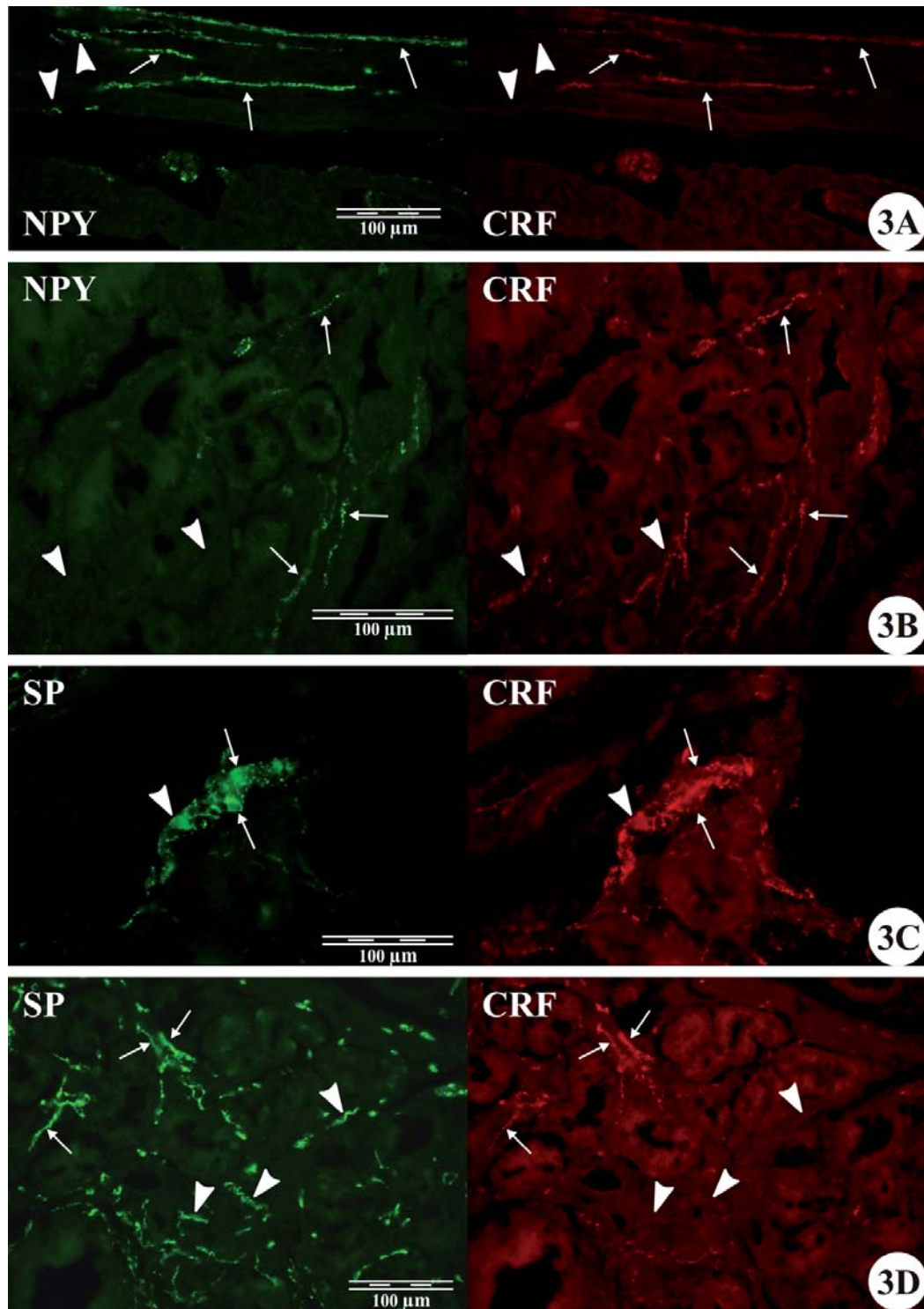


Figure 3. Cryostat sections of the ovine jejunum immunostained for CRF/NPY and CRF/SP. In the circular smooth muscle layer (3A) nerve fibres immunoreactive to CRF and NPY (arrows) and immunopositive to NPY but not CRF (arrowheads) are seen. In sections of the mucous layer of the ovine jejunum (3B) arrows indicate CRF-IR/NPY-IR nerve fibres, whereas arrowheads mark nerve fibres exhibiting the presence of CRF only (NPY-negative). Panel (3C) presents a submucous neuron co-localizing CRF and SP (arrowhead) as well as neurons expressing SP but not CRF (arrows); note the presence of CRF-expressing/SP-expressing nerve fibres in the submucous ganglia. (3D) CRF-IR/SP-IR nerve fibres were frequently observed between mucosal glands (arrows); arrowheads represent nerve fibres showing the presence of SP only (negative to CRF)

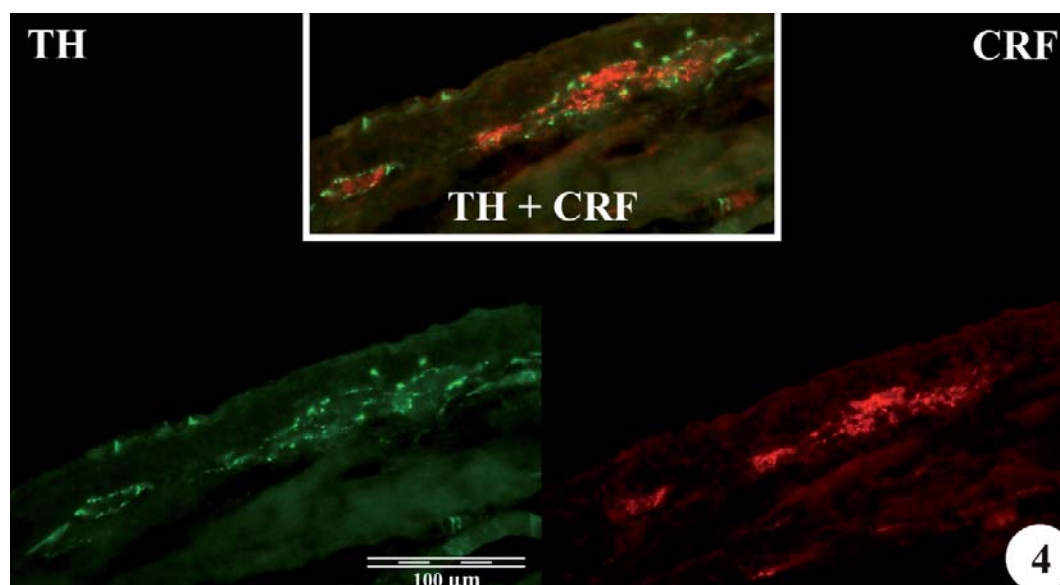


Figure 4. In the ovine jejunum CRF and TH do not co-localise in enteric nervous structures which is seen in detail in the merged picture

In the jejunum of the sheep neither CRF-IR enteric neurons nor CRF-positive nerve fibres located in the enteric ganglia and the circular smooth muscle and mucosal layers showed any presence of TH (Figure 4).

## DISCUSSION

The results presented herein show that CRF is widely distributed in the ovine jejunum which partially confirms previous radioimmunoassay studies conducted by Kawai et al. (1985). Immunohistochemistry revealed that CRF is expressed both in myenteric as well as submucous neurons and CRF-expressing nerve fibres supply enteric ganglia, the circular smooth muscle layer and the mucous layer. As we indicate in this study, in the ovine jejunum CRF-IR myenteric neurons constitute a minor population of enteric neurons (approx. 5%), which is in agreement with the results of previous studies. In the rat ileum only a small number of CRF-IR (up to 5%) myenteric neurons were found (Sand et al., 2011), and in the guinea-pig small intestine only 2.0 CRF-positive neurons per myenteric ganglion (approx. 2%) were counted (Liu et al., 2006). We also revealed that in the ovine jejunum, a relatively small population of submucous neurons (approx. 10%) expressed CRF; these data differ from those obtained in rodents. In the ileum of the rat up to 50% of submucous neurons expressed CRF (Sand et al., 2011). Also in the duodenum, jejunum and ileum

of the guinea-pig as many as 29.9%, 32.9% and 33.3% (respectively) of submucous neurons expressed CRF (Liu et al., 2006). In general, the distribution pattern of CRF-positive nerve fibres of the ovine jejunum resembles those observed in the small intestine of other mammals. Similarly to sheep, in the rodent small intestine a rich network of CRF-expressing nerve fibres was detected in both the myenteric and submucous plexuses (Wolter, 1984; la Fleur et al., 2005; Liu et al., 2006; Sand et al., 2011). Sand et al. (2011) also reported that the circular smooth muscle layer and mucous layer of the rat and guinea-pig small intestine contain moderate numbers of CRF-expressing nerve fibres. However, in the above mentioned species sparse CRF-IR nerve fibres were also detected in the longitudinal smooth muscle layer (Sand et al., 2011), as well as around submucosal arterioles (Liu et al., 2006; Sand et al., 2011), which is contradictory to our results.

Double immunohistochemistry revealed no co-localization of TH with CRF in nerve fibres supplying the ovine jejunum which is in line with a previous study of Liu et al. (2006), and indicates that no CRF-expressing nerve fibres are of extrinsic sympathetic origin. However, in the ovine jejunum CRF-expressing enteric neurons as well as CRF-IR nerve fibres widely co-stored biologically active substances (neuropeptides). So far, the presence of adrenocorticotropin and  $\beta$ -endorphin (but not Met-enkephalin) in CRF-containing myenteric and submucous neurons was reported in the rat duo-



denum (Wolter, 1985). It has also been shown that CRF in the guinea-pig small intestine is present in only a small population of cholinergic and nitrergic neurons (both myenteric and submucous), and no expression of CRF was found in those nervous structures immunoreactive to NPY, serotonin, somatostatin, calbindin and calretinin (Liu et al., 2006). On the other hand, the co-localization of CRF with SP and VIP was reported in the ENS of the guinea-pig and rat (Liu et al., 2006; Sand et al., 2011), which is consistent with our studies. However, we found that in the ovine jejunum nearly all CRF-IR myenteric and CRF-IR submucous neurons co-express VIP, whereas in the rat ileum 66% of myenteric neurons as well as 74% of submucous neurons were CRF-IR/VIP-IR. Basing on the distribution pattern of nerve fibres co-localizing CRF and VIP it is likely that in the ovine jejunum CRF-IR/VIP-IR myenteric neurons act as inhibitory circular smooth muscle motor neurons as well as descending interneurons. Support for the latter role may be provided by the findings that in the small intestine of the guinea-pig CRF stimulates  $Ca^{2+}$  transient in cholinergic myenteric neurons via CRF1 receptors (Bisschops et al., 2006) and enteric neurons of the rat small intestine express, besides CRF1, CRF2 receptors (Porcher et al., 2005). The co-incidence of CRF with VIP in myenteric neurons may also have implications for neuronal survival especially as in cultured myenteric neurons CRF counteracts the neuroprotective effect of VIP (Sand et al., 2011). Of note, in the guinea-pig small intestine all CRF-IR submucous neurons were reported to co-store VIP (no data concerning myenteric neurons were given), which prompted the authors to suggest that all CRF-expressing submucous neurons are VIP-ergic secretomotors (Liu et al., 2006). This claim may be strengthened by the finding that in the ovine jejunum substantial numbers (20%) of CRF-expressing submucous neurons co-stored galanin. In the mammalian small intestine VIP-IR/galanin-IR submucous neurons are considered as non-cholinergic secretomotor/vasodilator neurons which innervate mucosal glands and myenteric ganglia (Furness, 2000). Galanin is a strong constrictor of intestinal smooth muscles (Ekblad et al., 1985) and the lack of co-incidence between CRF and galanin in the circular smooth muscle layer may suggest that in the sheep jejunum galanin is not involved in the observed CRF-mediated alterations of small intestine contractility (la Fleur et al., 2005). However, since numerous CRF-IR/galanin-IR nerve fibres

were noted in the lamina muscularis mucosae of the ovine jejunum it is possible that both neuropeptides functionally co-operate in the generation of the local movement of the mucosa.

Double immunohistochemical studies indicated that in the ovine jejunum NPY is present both in CRF-expressing myenteric as well as submucous neurons. Of note, in the guinea-pig small intestine no coincidence of CRF with NPY in either enteric neurons or nerve fibres was observed (Liu et al., 1996). Since we discovered numerous CRF-IR/NPY-IR nerve fibres in the circular smooth muscle layer and myenteric ganglia it is likely that these nerve fibres originate from CRF-IR/NPY-IR myenteric neurons. Therefore CRF-IR/NPY-IR myenteric neurons probably act as inhibitory circular motor neurons and descending interneurons. We base this conclusion on the fact that NPY was very frequently observed in CRF-expressing myenteric neurons which means that the vast majority (if not all) of CRF-IR/NPY-IR myenteric neurons also co-store VIP. The co-localisation of VIP with NPY is commonly noted in short inhibitory circular motor neurons as well as controlling local reflexes descending interneurons (Costa et al., 1996). Additionally, we found that CRF-IR/NPY-IR nerve fibres supply the mucous layer of the ovine jejunum and in the light of recent findings it is likely that these nerve fibres may originate from secretoneurons which have located their cell bodies both in myenteric and submucous ganglia (Brookes, 2001). In previous pharmacological studies it was found that in the rat jejunum NPY evokes strong anti-secretory effects (Cox and Cuthbert, 1990). Since in the rat small intestine endogenous release of CRF mediates intestinal secretory and motor responses elicited by stress (Lenz et al., 1988), one could speculate that in the ovine jejunum NPY may functionally counteract the secretory effect of CRF.

Our findings indicate that SP is also expressed in minor subpopulations of CRF-IR myenteric neurons as well as CRF-IR submucous neurons (10% and 5%, respectively). This is in contrast to a previous study of Liu et al. (2006), who reported that in the small intestine of the guinea-pig 55% of CRF-expressing myenteric neurons were also SP-positive and no co-localization of CRF with SP was detected in submucous neurons. It is noteworthy that in the murine ileum, some CRF-IR/SP-IR enteric nerves were found (Anton et al., 2004). In the mammalian intestine SP-expressing myenteric neurons projecting into the circular smooth muscle layer are considered to be cholinergic excitatory motor neurons (Brookes, 2001).



Lazar et al. (2003) reported that CRF caused stimulation of indomethacin-treated circular muscle strips of the guinea-pig ileum and this effect was mediated by excitatory neurons of the myenteric plexus. Also, in the rat colon peripheral injection of CRF evoked neurally-mediated increases in colonic motility as well as increases in mucous secretion (Castagliuolo et al., 1996). Thus, it is likely that in the ovine jejunum CRF can both contract and relax the circular smooth muscles. Additionally, SP is a major neurotransmitter of intrinsic primary afferent neurons (IPAN) which suggests that at least a portion of SP-IR/CRF-IR enteric neurons may also be sensory in nature. Admittedly, Lazar et al. (2003) suggested that capsaicin-sensitive neurons do not mediate the effect of exogenous CRF in the guinea-pig ileum but other reports have indicated that CRF is an important factor involved in visceral pain development during inflammatory processes (for a review see Larauche et al., 2009). Since in the wild type mouse, the co-localization of CRF with SP in the ileal enteric nerves is more evident after *Clostridium difficile* toxin A administration it has been proposed that CRF secreted in response to toxin A is correlated with the pro-inflammatory effect of SP (Anton et al., 2004).

In summary, this work for the first time indicates the presence of CRF in the ENS of the jejunum of the sheep. CRF-expressing myenteric and submucous neurons as well as CRF-IR nerve fibres located in particular layers of the ovine jejunum contain varying amounts of neuropeptides. The frequency and distribution pattern of CRF-expressing enteric nerves, suggest that in the jejunum of the sheep CRF plays a regulatory role(s). Analysis of the chemical coding of CRF-IR enteric neurons indicates that VIP, galanin, SP and NPY may be functionally involved in CRF-mediated regulation of intestinal activities.

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