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# Neuroimmunomodulative Properties of Dipeptidyl Peptidase IV/CD26 in a TNBS-Induced Model of Colitis in Mice

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## ABSTRACT

Causal connections between dipeptidyl peptidase IV, also known as CD26 molecule (DPP IV/CD26) and inflammatory bowel disease (IBD) have been shown, but mechanisms of these interactions are unclear. Our hypothesis was that DPP IV/CD26 could affect the neuroimmune response during inflammatory events. Therefore, we aimed to evaluate its possible role and the relevance of the gut–brain axis in a model of IBD in mice. Trinitrobenzenesulfonic acid-induced (TNBS) colitis was induced in CD26-deficient ($CD26^{-/-}$) and wild-type (C57BL/6) mice. Pathohistological and histomorphometrical measurements were done. Concentrations and protein expressions of DPP IV/CD26 substrates neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) were determined. Concentrations of IL-6 and IL-10 were evaluated. Investigations were conducted at systemic and local levels. Acute inflammation induced increased serum NPY concentrations in both mice strains, more enhanced in $CD26^{-/-}$ mice. Increased NPY concentrations were found in colon and brain of C57BL/6 mice, while in $CD26^{-/-}$ animals only in colon. VIP and IL-6 serum and tissue concentrations were increased in both mice strains in acute inflammation, more pronouncedly in $CD26^{-/-}$ mice. IL-10 concentrations, after a decrease in serum of both mice strains, increased promptly in $CD26^{-/-}$ mice. Decreased IL-10 concentration was found in brain of C57BL/6 mice, while it was increased in colon of $CD26^{-/-}$ mice in acute inflammation. DPP IV/CD26 deficiency affects the neuroimmune response at systemic and local levels during colitis development and resolution in mice. Inflammatory changes in the colon reflected on investigated parameters in the brain, suggesting an important role of the gut–brain axis in IBD pathogenesis. J. Cell. Biochem. 112: 3322–3333, 2011. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** DIPEPTIDYL PEPTIDASE IV/CD26; CD26-DEFICIENT MICE; TNBS-INDUCED COLITIS; NEUROPEPTIDE Y; VASOACTIVE INTESTINAL PEPTIDE; INTERLEUKIN-6; INTERLEUKIN-10

Inflammatory bowel disease (IBD) comprises two main chronic pathologies of the gastrointestinal tract (GIT): ulcerative colitis (UC) and Crohn's disease (CD), both characterized by alternating phases of active inflammation and clinical remission with different complications and extraintestinal manifestations [Hanauer and Hommes, 2010]. The ethiopathogenesis of IBD has still not been elucidated, but it has been suggested that inflammatory processes emerge in genetically susceptible individuals as a result of an irregular, over-expressed immunological reaction to some undefined food antigens or some other agents of microbial origin [Blumberg, 2009]. However, there is no therapeutic approach which could finally make this debilitating disease curable.

In the last few years, a very dynamic field of investigation in IBD ethiopathogenesis is neuroimmunobiochemistry, whose hypothesis is supported by a growing body of evidence that neurogenic inflammation could be a key event in inflammatory mechanisms in the GIT [Takami et al., 2009]. This is a consequence of the bidirectional communication between the central and enteric nervous system and, therefore, a term "gut–brain axis" has been introduced and proposed to play an important role in the etiology of gut inflammation [Romijn et al., 2008].

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Given the complexity of etiological factors in human IBD, a lot of current knowledge regarding IBD pathogenesis has arisen from the study of various animal models [Mizoguchi and Mizoguchi, 2010]. Although no ideal model of IBD has been accomplished so far, they resemble different important clinical, histopathological, and immunological aspects of human IBD, and therefore represent essential tools in investigating mechanisms underlying inflammation in the GIT [Strober, 2008]. Chemically induced murine models of IBD are the most commonly used due to their onset and duration of inflammation which is immediate, reproducible, and shares a lot of similarities with human IBD in multiple aspects, including cytokine deregulations and production of inflammatory mediators [Mizoguchi and Mizoguchi, 2010]. Trinitrobenzenesulfonic acid-induced colitis (TNBS-colitis) is one of the most accepted and used CD models in mice and has given insight in many processes at the molecular level [Wirtz and Neurath, 2007].

Proteases are proposed as one of the key factors in the occurrence of inflammatory processes due to their ability to metabolize different biologically active molecules implicated in maintaining the integrity of mucosal barrier [Detel et al., 2007; Ravi et al., 2007], one of which is dipeptidyl peptidase IV, also known as CD26 molecule (DPP IV/CD26) [Gorrell et al., 2006]. DPP IV/CD26 is also a T-cell differentiation antigen, expressed on various cell types, having numerous functions in a variety of biological processes, as well as immunological mechanisms. It is also present in a soluble form which circulates in body fluids of living organisms, with a specific peptidase function: it cleaves dipeptides from the N terminus of polypeptides having proline or alanine at the penultimate position [Vanderheyden et al., 2009]. Since Xaa-Pro peptides are not easily metabolized by other proteases, the action of DPP IV/CD26 is an essential step in the degradation of many polypeptides [Matteucci and Giampietro, 2009].

Numerous biologically important cytokines, chemokines, and neuropeptides with potential and/or confirmed role in IBD ethiopathogenesis are effective DPP IV/CD26 substrates [Mentlein, 2004]. Vasoactive intestinal peptide (VIP) is a peptide hormone produced in many areas of the human body including the gut, pancreas, and brain [Delgado et al., 2004]. It has recently been proposed as a healing mediator in CD, since VIP treatment promoted the recovery of clinical symptoms, the amelioration of parameters correlated to the recruitment and traffic of cell populations, and the balance of inflammatory mediators derived from granulocytes, antigen-presenting cells, and T lymphocytes comprising Th1, Th2, and Th17 response in a TNBS model of colitis in mice [Arranz et al., 2008]. Neuropeptide Y (NPY) is a gut–brain peptide, found in different parts of living organisms, including central and enteric nervous system [Gehlert, 2004]. Previous findings proved its involvement in the pathogenesis of IBD, but its role is controversial and not entirely clear. It has been shown that targeted deletion of NPY modulates experimental colitis and that NPY and its receptor Y1 are involved in processes of intestinal inflammation [Hassani et al., 2005; Chandrasekharan et al., 2008]. DPP IV/CD26 cleaves NPY and changes its affinity for Y1 receptor which has been suggested to play an important role in inflammatory mechanisms leading to colitis development [Dimitrijevic et al., 2008]. In addition, a causal connection between NPY, VIP, and interleukins of critical importance for IBD, such as IL-6 and IL-10, has been proved [Delgado et al., 2004; Gehlert, 2004].

Previous studies from several research groups, including ours, showed a correlation between IBD disease severity and serum DPP IV/CD26 activity [Hildebrandt et al., 2001; Varljen et al., 2005]. Furthermore, a number of studies proposed a potential role of DPP IV/CD26 in the pathogenesis of IBD, given its involvement in immune regulations via its expression on immune cells and capability to cleave biologically active molecules [Matteucci and Giampietro, 2009; Reinhold et al., 2009]. Additionally, DPP IV/CD26 inhibitors have been pointed out as therapeutic agents in ameliorating inflammatory processes in immunologically mediated diseases such as IBD, however, further research is needed in this field [Bank et al., 2008; Yazbeck et al., 2009].

Given the potential role of DPP IV/CD26 and possible involvement of neurogenic inflammation in the pathogenesis of IBD, the aim of this study was to investigate does DPP IV/CD26 and in which manner affect the neuroimmune response during development, progression, and resolution of inflammatory events in a Crohn-like model of colitis in mice. Furthermore, we wanted to evaluate the relevance of the gut–brain axis in the development of inflammatory processes and tissue healing.

## MATERIALS AND METHODS

### EXPERIMENTAL ANIMALS

The study comprised two mice strains: wild-type mice strain C57BL/6 and mice with inactivated gene for molecule CD26 (C57BL/6 Jbom-ob, CD26$^{-/-}$), generated on a C57BL/6 genetic background as described previously [Marguet et al., 2000]. CD26$^{-/-}$ mice were kindly provided by Dr. Didier Marguet (Centre d'Immunologie Marseille-Luminy, France). Male, 8- to 10-week-old mice, group-housed and bred under conventional conditions at the Central Animal Facility of the School of Medicine (University of Rijeka) were used in this study. Laboratory animals were housed in plastic cages, fed with standard pellet food (MK, Complete Diet for Laboratory Rats and Mice, Slovenia), given tap water ad libitum and maintained under a 12/12 h dark/light cycle at constant temperature ($20 \pm 1$)°C and humidity ($50 \pm 5$)%. Each study group comprised 8–10 animals. Handling laboratory animals, experimental procedures, and anesthesia were performed following general principles contained in the Guide for the Care and Use of Laboratory Animals (National Academic Press). The Ethical Committee of the School of Medicine (University of Rijeka), approved all experimental procedures.

### COLITIS INDUCTION

Crohn-like colitis (TNBS-colitis) was induced by rectal administration of 5% (w/v) TNBS (Sigma–Aldrich, Germany) dissolved in 50% ethanol (Kemika, Croatia). Each animal received 0.1 ml of TNBS–ethanol solution, using a vinyl catheter that was positioned 4 cm from the anus, as described in literature [Scheiffele and Fuss, 2002]. Two control groups of mice were used for each mice strain. Control mice underwent indistinguishable procedures, but were instilled equal volumes of saline (NaCl 0.9%) or ethanol solution. Prior to administration of TNBS, saline, or ethanol solution, mice were anesthetized with a combination of ketamine/xylazine solution.

### EXPERIMENTAL PROCEDURES

Mice body weights and overall clinical characteristics were monitored daily. Laboratory animals were sacrificed by cervical dislocation after 2, 7, 15, and 30 days following administration of TNBS, saline, or ethanol solution. Peripheral blood samples were taken and underwent centrifugation at 3,000 rpm for 10 min in order to collect serum samples. Liver and spleen were isolated, washed in ice-cold saline, and weighted. The entire GIT was isolated, carefully examined, the colon was freed from adhering connective and adipose tissue and macroscopic changes were noted. Colonic lumens were carefully washed with ice-cold saline and their weights and lengths were measured. One part of the colon was separated for further histological procedures and analyses, and another part was used for protein extraction. Immediately after sacrifice and skull dissection, both brain hemispheres were taken, washed in ice-cold saline, and separately frozen in liquid nitrogen. Samples were stored at $-80^{\circ}$C until further analyses.

### PATHOHISTOLOGICAL EVALUATION AND HISTOMORPHOMETRIC MEASUREMENTS

Colon tissues were fixed in 4% formalin for 24 h; samples were processed and embedded in paraffin wax. Two micrometers tissue sections were stained with hematoxylin and eosin and analyzed by an experienced pathologist, blinded to treatment allocation. Microscopical changes including overall severity of damage, number of crypts of Lieberkuhn, and their depth and width were evaluated. Results were expressed as mean $\pm$ SD. Microscopic analysis was carried out using an Olympus BX40 microscope (Tokyo, Japan) and the Pulnix TMC 76S digital camera (Tokyo, Japan). Analyses of digital images were performed using Issa software package (VAMS, Zagreb, Croatia).

### ELISA AND EIA ANALYSES

ELISA and EIA analyses were performed in order to quantify levels of interleukins and neuropeptides in mouse serum, colon, and brain homogenates in determined time intervals during colitis development and resolution. In order to obtain tissue homogenates, brains and colons were homogenized on ice in a 0.5 M acetic acid solution with addition of commercially available inhibitors of proteases an phosphatases (Santa Cruz Biotechnology Inc., CA), according to the method of El-Karim et al. [2003]. The volume of peptide extraction solution used in all extraction procedures was 8 ml/g of tissue. Samples were heated at 95°C for 10 min then centrifuged at 14,000 rpm for 20 min at $+4^{\circ}$C. Resulting supernatants were measured for total protein concentrations according to the method of Bradford [1976]. Samples were aliquoted before being stored at $-80^{\circ}$C.

Concentrations of neuropeptides NPY and VIP and interleukins IL-6 and IL-10 in serum and tissue homogenates were determined according to the manufacturer's instructions (NPY and VIP EIA kits; Phoenix Pharmaceuticals, Belmont, CA; IL-6 and IL-10 ELISA kits, R&D Systems, Europe, UK). Results of measurements in serum samples are expressed as mean $\pm$ SEM per ml of serum and as pg/mg of total protein in tissue homogenates.

### WESTERN BLOT ANALYSES

Protein expressions of NPY and VIP were determined in brain and colon tissues. Samples were homogenized on ice using RIPA lysis buffer including inhibitors of proteases and phosphatases (Santa Cruz Biotechnology Inc.). Homogenates were then centrifuged at 14,000 rpm for 20 min at $+4^{\circ}$C and resulting supernatants were measured for total protein concentrations according to the method of Bradford [1976]. Samples were then heated at 95°C for 5 min in a sample buffer mixture.

Equal amounts of total proteins (50 μg/lane) were separated by SDS–PAGE on 12.5% gels. Prestained molecular weight markers (Kaleidoscope Prestained Standards, BIO-RAD and Fermentas Life Sciences PageRuler$^{TM}$) were used as standards. Samples were electrophorized at 50 V for 6 h on ice. Proteins were transferred from polyacrylamide gels to polyvinylidenedifluoride membranes by electroblotting technique in a semi-dry transfer device at 0.22 mA for 45 min. Nonspecific sites of antibody recognition were blocked by immersing membranes for 2 h at 4°C in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T, Amersham Biosciences), containing 5% (w/v) nonfat milk powder (Santa Cruz Biotechnology Inc.). Membranes were incubated overnight with primary anti-NPY and anti-prepro-VIP antibody (Santa Cruz Biotechnology Inc., 1:200 in blocking buffer). Blots were washed three times for 15 min in PBS-T. Horseradish peroxidase-conjugated mouse-anti-rabbit IgG were used as secondary antibodies, in a dilution of 1:2,000 (Santa Cruz Biotechnology Inc.). After washing procedure, NPY and VIP were detected by a two-compound system chemiluminescent, Amersham ECL-plus Western blotting detection reagent (Amersham, Little Chalfont, UK), which enabled visualization of bends after exposure to photosensitive films (AGFA Ortho CP-G plus).

Equal amounts of total protein loading were controlled by using primary mouse ß-actin antibody (Chemicon International), in a dilution of 1:40,000, and secondary horseradish peroxidase-conjugated goat-anti-mouse IgG in a dilution of 1:2,000 (Santa Cruz Biotechnology Inc.).

### STATISTICAL ANALYSES

Statistical comparisons were made using STATISTICA version 8.0 (StatSoft Inc., Tulsa). Differences between groups have been tested using ANOVA, followed by post hoc Scheffé test. The level of $P < 0.05$ was considered statistically significant.

## RESULTS

### COLITIS EVALUATION

Systemic features associated with intrarectal administration of TNBS solution included poor clinical state, body weight loss up to 15%, and a mortality rate of approximately 11% in both CD26$^{-/-}$ and C57BL/6 mice which was mostly pronounced in the first 5 days of experiment. Macroscopic inspection of the distal part of the colon revealed mucosal erosions, congestion, localized inflammation, and some ulceration. Symptoms of inflammation were mostly prominent the 2nd day following TNBS administration and additionally comprised: noticeable macroscopic inflammation localized in the distal part of the colon, colon shortening and thickening, marked colonic edema, and presence of hemorrhagic changes (Fig. 1B,C).

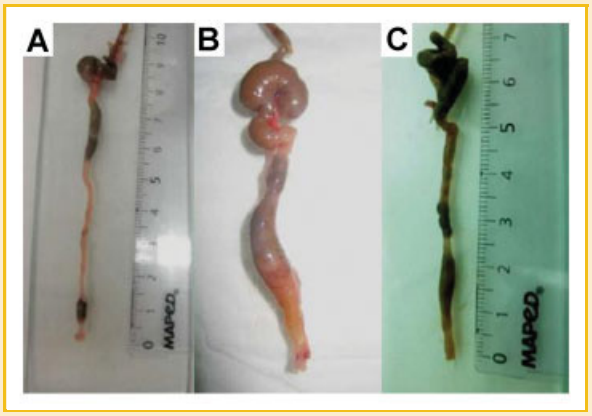


Fig. 1. Macroscopic appearance of colons harvested from (A) CD26$^{-/-}$ control mice, and TNBS-treated CD26$^{-/-}$ mice in the acute phase of inflammation, 2 days after administration of TNBS solution (B,C). Colon shortening and thickening, marked colonic edema, and presence of hemorrhagic changes could be seen. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Therefore, the 2nd day following TNBS administration was classified as acute phase of colitis, which is in accordance with previously reported findings [Scheiffele and Fuss, 2002].

Pathohistological analyses confirmed the presence of inflammatory changes in colons of both CD26$^{-/-}$and C57BL/6 mice. Intraluminal infusion of a TNBS–ethanol solution in both mice strains induced an acute granulomatous, transmural inflammation with infiltration of inflammatory cells mainly located in the distal part of the colon, which resembles human CD with regard to histological and immunological features as previously described in literature [Wirtz and Neurath, 2007]. The typical sign of early state of disease was focal mucosa ulceration, along with predominantly neutrofilic infiltration in lamina propria, fibrin deposition, and submucosal edema (Fig. 2B,C). In the ulcerated tissue, abundant hemorrhage with some necrotic part of mucosa without any crypt residue or epithelial layer could be seen (Fig. 2D). With disease progression, further destruction of the mucosa leads to outright ulceration, extending into submucosa and deeply through bowel wall leading to serositis. During colitis resolution and tissue healing, architectural crypt distortion with mononuclear inflammatory cells infiltration in lamina propria as well as submucosa could be seen (Fig. 2E,F). Necrotic parts of tissue were replaced with mild fibrosis in lamina propria (Fig. 2G).

Results of histomorphometrical analyses confirmed the presence of inflammatory changes in both CD26$^{-/-}$and C57BL/6 mice that received TNBS–ethanol solution. Number of crypts of Lieberkuhn per mm of mucosa, their depth and width for different groups of both mice strains at specified days of experiment were measured (Table I). A statistically significant ($P < 0.05$) decrease in number of crypts of Lieberkuhn per mm of mucosa was observed in the acute phase of colitis in both mice strains with induced colitis. Changes persisted during tissue healing in CD26$^{-/-}$ mice. The widths of Crypts of Lieberkuhn were increased in the acute phase of colitis in both mice strains, but in C57BL/6 mice the reappearance of physiological values was slower. Finally, the depth of Crypts of Lieberkuhn was

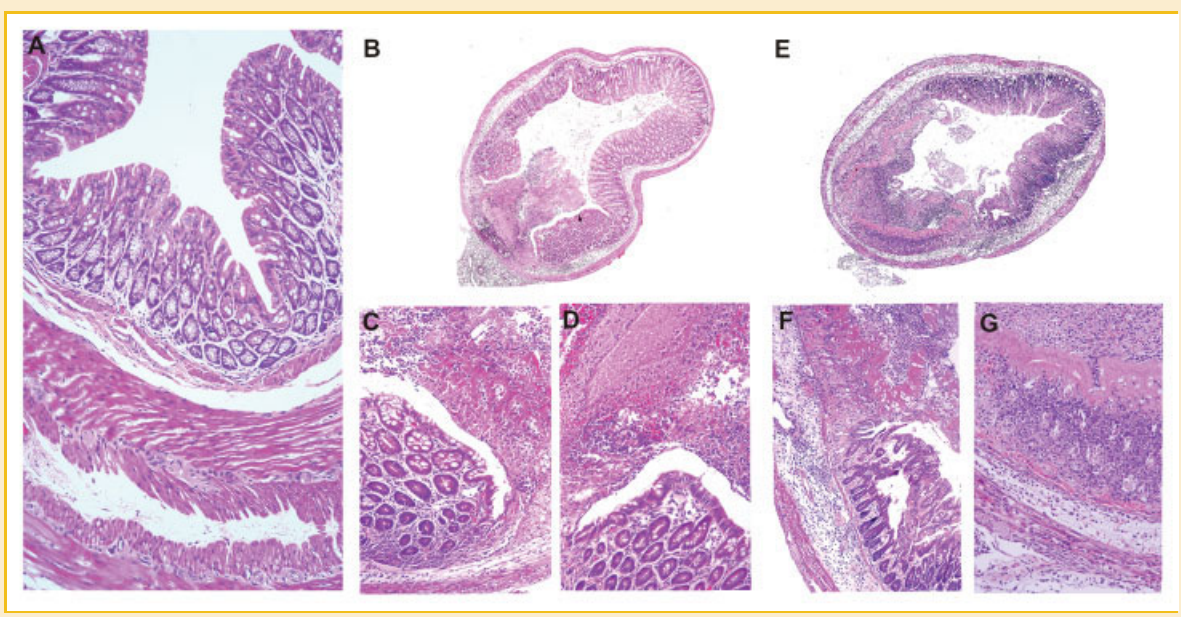


Fig. 2. Histopathologic examination of H&E-stained paraffin colonic tissues sections from CD26$^{-/-}$ mice. A: Normal colon of control mouse. B–D: TNBS-instilled colons in acute inflammation. Mucosal ulceration, associated with predominantly neutrofilic infiltration in lamina propria, fibrin deposition, and submucosal edema. Abundant hemorrhage with some necrotic part of mucosa without any crypt residue or epithelial layer could be seen. E–G: Colon sections 7 days after administration of TNBS solution. Architectural crypt distortion with mononuclear inflammatory cells infiltration in lamina propria and submucosa could be seen. Necrotic parts of tissue replaced with mild fibrosis in lamina propria are observed. Magnifications: (A,D,G) 20×; (C,F) 10×; (B,E) 4×. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Results of Histomorphometrical Analyses for Different Groups of TNBS-Treated and Ethanol-Treated (Control Groups) of CD26-Deficient ($CD26^{-/-}$) and Wild-Type (C57BL/6) Mice at Specified Day of Experiment

| | | Time (days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 2 | | 7 | | 15 | | 30 | | |
| Parameter | Mice strain | Physiological | Colitis | Control | Colitis | Control | Colitis | Control | Colitis | Control |
| Crypts number (N/mm of mucosa) | $CD26^{-/-}$ | $26.40 \pm 2.48$ | $9.05 \pm 3.29^{a,b}$ | $20.29 \pm 2.75$ | $11.91 \pm 1.11^{a,b}$ | $21.54 \pm 4.02$ | $15.77 \pm 3.04^{a,b}$ | $25.11 \pm 5.21$ | $16.27 \pm 2.31$ | $24.67 \pm 6.46$ |
| | C57BL/6 | $25.90 \pm 3.94$ | $13.88 \pm 2.05^{a,b}$ | $20.82 \pm 1.52$ | $14.14 \pm 2.53^{a,b}$ | $22.61 \pm 5.10$ | $22.25 \pm 5.69$ | $23.81 \pm 5.31$ | $22.09 \pm 4.12$ | $23.37 \pm 7.12$ |
| Crypt depth (μm) | $CD26^{-/-}$ | $220.13 \pm 44.02$ | $118.22 \pm 30.14^{a,b}$ | $209.43 \pm 42.91$ | $178.53 \pm 47.85$ | $213.85 \pm 53.15$ | $202.56 \pm 45.22$ | $212.08 \pm 57.25$ | $212.12 \pm 45.11$ | $222.88 \pm 38.78$ |
| | C57BL/6 | $229.97 \pm 36.11$ | $119.31 \pm 32.83^{a,b}$ | $197.67 \pm 36.63$ | $198.67 \pm 44.17$ | $222.10 \pm 34.74$ | $210.56 \pm 38.18$ | $229.31 \pm 30.63$ | $233.33 \pm 35.54$ | $229.40 \pm 35.72$ |
| Crypt width (μm) | $CD26^{-/-}$ | $27.41 \pm 8.49$ | $88.77 \pm 26.44^{a,b}$ | $39.14 \pm 9.34$ | $44.25 \pm 7.78$ | $33.60 \pm 8.84$ | $41.97 \pm 16.65$ | $31.50 \pm 13.66$ | $32.83 \pm 11.67$ | $28.20 \pm 11.22$ |
| | C57BL/6 | $28.48 \pm 6.95$ | $80.23 \pm 23.39^{a,b}$ | $38.05 \pm 17.10$ | $57.87 \pm 21.62^{a,b}$ | $37.93 \pm 11.76$ | $34.90 \pm 14.27$ | $28.58 \pm 8.73$ | $34.48 \pm 9.45$ | $29.16 \pm 9.92$ |

[a]Statistically significantly different compared to physiological values ($P < 0.05$).
[b]Statistically significantly different compared to related control group ($P < 0.05$).

decreased in the acute phase of colitis in both mice strains. Observed changes represent an outcome of inflammatory processes in the colon which comprise mucosa thickening and formation of edema due to tissue damage induced by TNBS solution. Histomorphometrical measurements confirmed characteristic inflammatory changes as found in IBD humans. Nevertheless, there were no statistically significant differences observed between $CD26^{-/-}$and C57BL/6 mice in analyzed histomorphometrical parameters. Statistical analyses of obtained histomorphometrical results among both control groups of animals did not reveal statistically significant changes in observed parameters.

### EFFECT OF CD26 DEFICIENCY ON NEUROPEPTIDES CONCENTRATIONS IN SERUM, COLON, AND BRAIN

Concentrations of neuropeptides VIP and NPY were determined at systemic and local levels, among the gut–brain axis, at different time points after colitis induction in $CD26^{-/-}$and C57BL/6 mice.

### CD26 DEFICIENCY CAUSES HIGHER VIP CONCENTRATIONS IN ACUTE INFLAMMATION

Changes in VIP concentration in mice serum, colon, and brain at specified time points of experiment are shown in Figure 3. It can be seen that $CD26^{-/-}$ mice constitutionally have statistically significantly ($P < 0.05$) higher serum VIP concentrations compared to C57BL/6 mice (Fig. 3A). VIP serum concentrations in both mice strains reach their maximum values in the acute phase of colitis. The increment in serum VIP concentration is more pronounced in $CD26^{-/-}$ mice, with an increase of 58.5% compared to physiological values. VIP serum concentrations are statistically significantly increased ($P < 0.05$) in acute inflammation in both mice strains compared to their controls, but VIP concentration remains statistically significantly higher ($P < 0.05$) only in $CD26^{-/-}$ mice 7 days after application of TNBS solution, when the process of healing starts. Serum VIP concentrations normalize to physiological values in later days of experiment, when clinical and histological signs of inflammation disappear.

No statistically significant differences in colon VIP content were found between mice strains in physiological conditions (Fig. 3B). However, under conditions of colon inflammation, VIP concentrations statistically significantly increase ($P < 0.05$) in the acute phase of colitis in both strains, compared to their control groups. Furthermore, VIP concentration in colon is statistically significantly ($P < 0.05$) higher in $CD26^{-/-}$ compared to C57BL/6 mice under conditions of acute inflammation. Like in serum, colon VIP concentrations remain statistically significantly higher ($P < 0.05$) only in $CD26^{-/-}$ mice 7 days after TNBS application, compared to their control group. Western blot analyses confirmed the results obtained by EIA, showing a higher level of VIP protein expression in colons during the acute phase of colitis compared to control groups in both mice strains (Fig. 4A).

Most prominent VIP increment in the brain was also seen in the acute phase of colitis (Fig. 3C). The increase in VIP concentration in brain is statistically significantly higher in both mice strains compared to their controls ($P < 0.05$), and it is higher in $CD26^{-/-}$ mice compared to C57BL/6 mice. Results of EIA were confirmed by Western blotting which showed an enhanced level of VIP protein

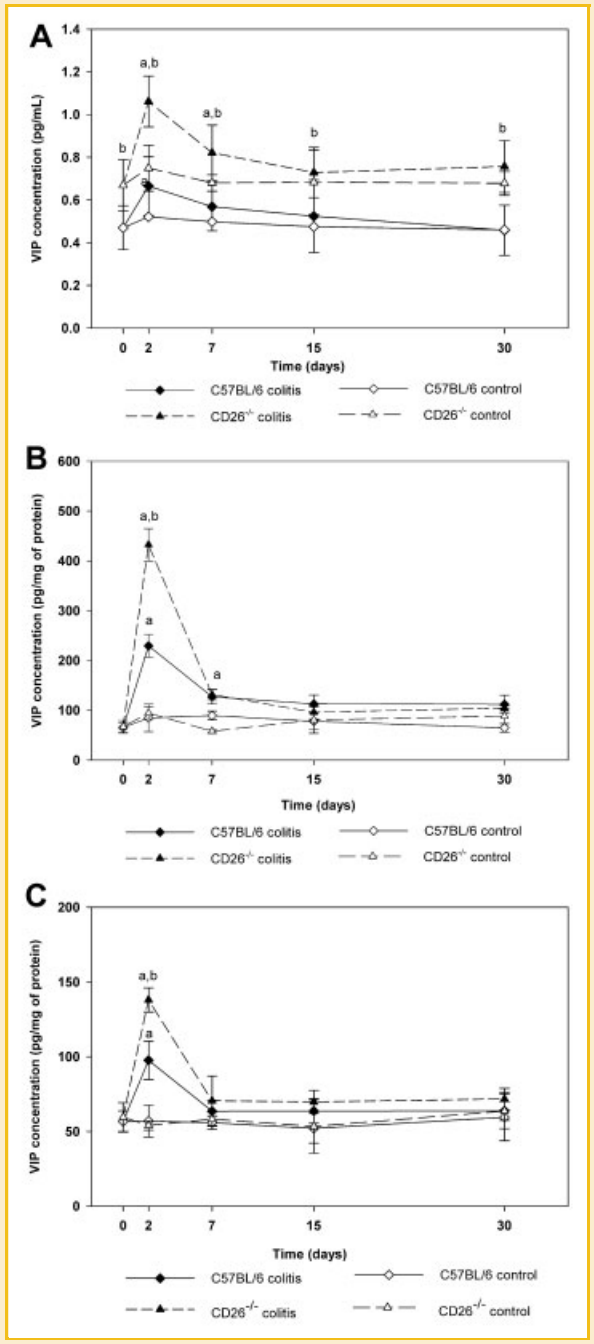


Fig. 3. (A) Serum, (B) colon, and (C) brain VIP concentrations at different time schedule during colitis development and resolution in CD26$^{-/-}$ and C57BL/6 mice. [a]Statistically significantly different ($P < 0.05$) from related control group. [b]Statistically significantly difference ($P < 0.05$) between CD26$^{-/-}$ and C57BL/6 mice.

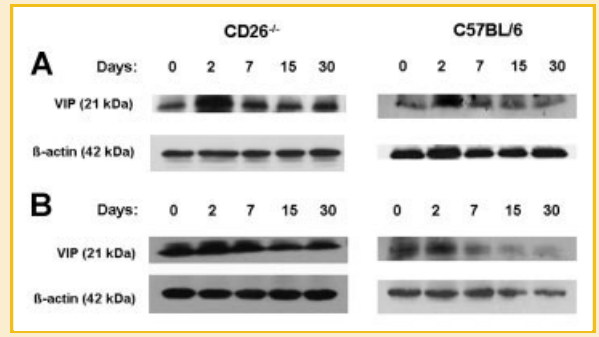


Fig. 4. Western blot detection of VIP protein expression in (A) colon and (B) brain of CD26$^{-/-}$ and C57BL/6 mice at different time schedule during colitis development and resolution. Equal total protein loading was controlled by the same level of detected β-actin protein expression in each group.

expression in brains in the acute phase of colitis, compared to control groups in both mice strains (Fig. 4B).

## CD26 DEFICIENCY AFFECTS NPY CONCENTRATIONS IN INFLAMMATORY PROCESSES

Serum, colon, and brain NPY concentrations at different time schedule during colitis development and resolution in CD26$^{-/-}$ and C57BL/6 mice and their controls are presented on Figure 5. CD26$^{-/-}$ mice were found to have slightly, but not statistically significantly higher serum NPY concentrations compared to C57BL/6 mice (Fig. 5A). Inflammatory changes in the colon lead to an increase in serum NPY concentrations in both mice strains, starting in the acute phase of colitis and reaching its maximum on the 7th day of experiment ($P < 0.05$) in CD26$^{-/-}$ mice, compared to control group. Changes are more emphasized in CD26$^{-/-}$ mice, where concentrations of serum NPY remain statistically significantly higher compared to their controls and to C57BL/6 mice ($P < 0.05$), even the 15th day after colitis induction, during colitis resolution.

In physiological conditions, colon NPY content did not differ statistically significantly between mice strains (Fig. 5B). Under conditions of colon inflammation, NPY concentrations statistically significantly increase ($P < 0.05$) in the acute phase of colitis in both CD26$^{-/-}$ and C57BL/6 mice compared to their control groups. Interestingly, NPY concentration in colon is statistically significantly ($P < 0.05$) higher in C57BL/6 compared to CD26$^{-/-}$ mice under conditions of acute inflammation and in the recovery phase. Results obtained by Western blotting confirmed an enhanced NPY expression in the acute phase of colitis in colons of both CD26$^{-/-}$ and wild-type mice. In C57BL/6 mice, the enhanced NPY protein expression persists longer, till the beginning of the recovery phase (Fig. 6A).

In physiological conditions, CD26$^{-/-}$ had slightly, but not statistically significantly higher NPY brain content (Fig. 5C). We noticed that CD26 deficiency causes opposite changes in brain NPY content during inflammatory processes compared to C57BL/6 mice. While colitis induces a statistically significant increase ($P < 0.05$) in NPY concentration in the acute phase in C57BL/6 mice, in CD26$^{-/-}$ mice a statistically significantly decrease ($P < 0.05$) of NPY concentration in brain has been determined the 2nd and 7th day following TNBS application, compared to their controls. On the other hand, a statistically significant decrease ($P < 0.05$) in brain NPY content was found in C57BL/6 mice in the period of colitis resolution, the 15th day after application of TNBS solution. Western blot analyses confirmed results obtained by EIA, showing decreased NPY protein expression in brain of CD26$^{-/-}$ mice the 2nd and 7th day after colitis induction, while it was increased in the acute phase in wild-type mice (Fig. 6B).

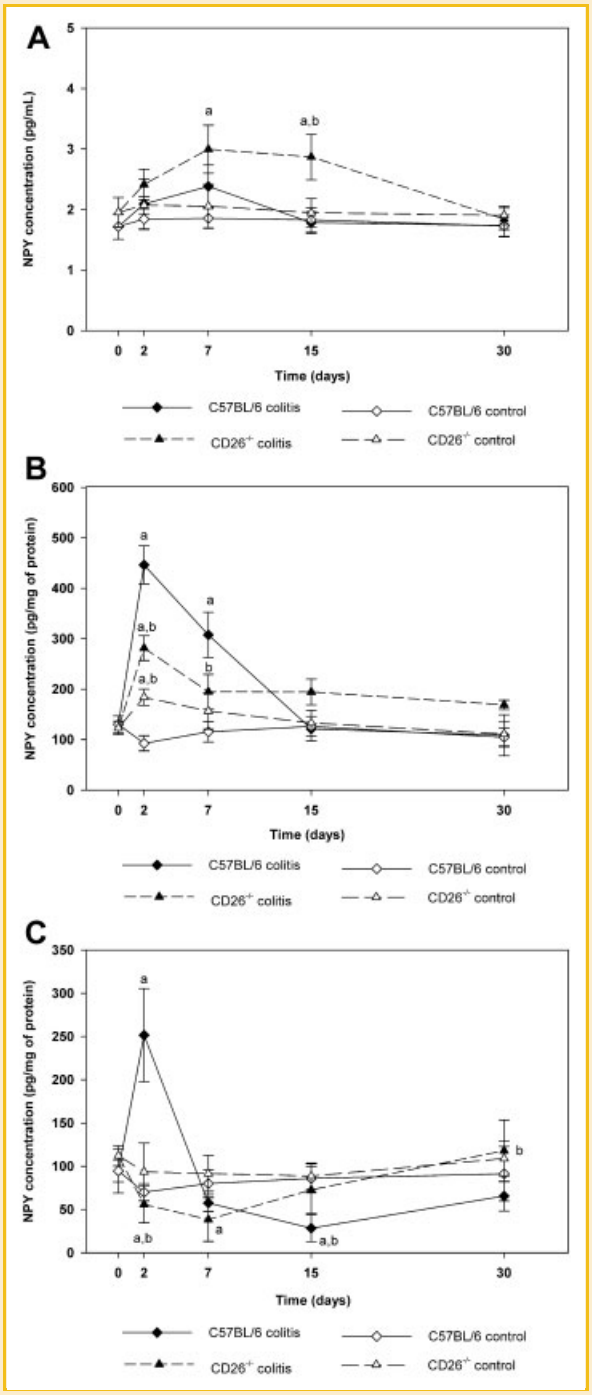


Fig. 5. (A) Serum, (B) colon, and (C) brain NPY concentrations at different time schedule during colitis development and resolution in CD26$^{-/-}$ and C57BL/6 mice. [a]Statistically significantly different ($P < 0.05$) from related control group. [b]Statistically significantly difference ($P < 0.05$) between CD26$^{-/-}$ and C57BL/6 mice.

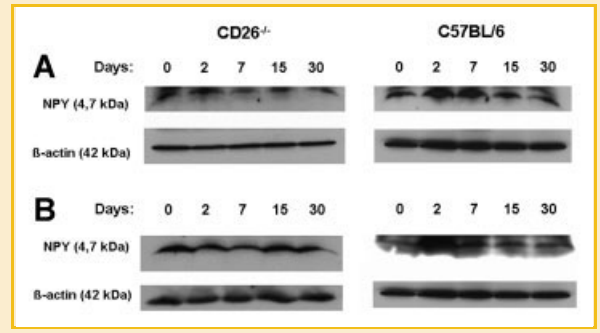


Fig. 6. Western blot detection of NPY protein expression in (A) colon and (B) brain of CD26$^{-/-}$ and C57BL/6 mice at different time schedule during colitis development and resolution. Equal total protein loading was controlled by the same level of detected β-actin protein expression in each group.

## EFFECT OF CD26 DEFICIENCY ON SERUM, COLON, AND BRAIN INTERLEUKINS CONCENTRATIONS

In order to have an insight in alterations of proinflammatory IL-6 and anti-inflammatory IL-10 at systemic (in serum) and local levels (among the gut–brain axis), their concentrations have been determined at scheduled time points during colitis development and tissue healing.

## CD26 DEFICIENCY AFFECTS IL-6 LEVELS

Figure 7 shows serum, colon, and brain IL-6 concentrations at different time points during colitis development and resolution in CD26$^{-/-}$ mice and their controls. CD26$^{-/-}$ mice were found to have constitutionally statistically significantly ($P < 0.05$) higher IL-6 concentrations in serum compared to C57BL/6 mice. Consequently, serum IL-6 concentrations remain statistically significantly higher during the entire experimental period in CD26$^{-/-}$ mice. The highest serum IL-6 concentrations have been determined in the acute phase of colitis in both mice strains (Fig. 7A).

On the other hand, CD26$^{-/-}$ and C57BL/6 mice did not statistically significantly differ in the content of IL-6 in colon in physiological conditions (Fig. 7B). A statistically significant increase ($P < 0.05$) in colon IL-6 concentrations at sites of inflammation has been found in both mice strains in the acute phase of inflammation compared to their controls. The increase was more pronounced in CD26$^{-/-}$ mice, but not statistically significantly.

As in case of neuropeptides, alterations of IL-6 concentrations in colon were followed by changes in IL-6 content in brain (Fig. 7C). CD26$^{-/-}$ mice had higher brain IL-6 concentrations compared to C57BL/6 mice in physiological conditions. The highest IL-6 concentrations in brain were found in both mice strains in the acute phase of colitis. Values decreased 7 days after TNBS solution administration in both mice strains, but interestingly, in CD26$^{-/-}$ mice the decrease was more accentuated and IL-6 concentrations reached statistically significantly lower ($P < 0.05$) concentrations compared to their physiological controls. Furthermore, those values were found to be statistically significantly lower ($P < 0.05$) also when compared to values found in C57BL/6 mice with induced colitis, on the same day of experiment.

## CD26 DEFICIENCY CAUSES CHANGES IN IL-10 CONCENTRATIONS

IL-10 concentrations in serum, colon, and brain at different time points during colitis development and resolution in CD26$^{-/-}$ and C57BL/6 mice and their controls are presented on Figure 8. As in

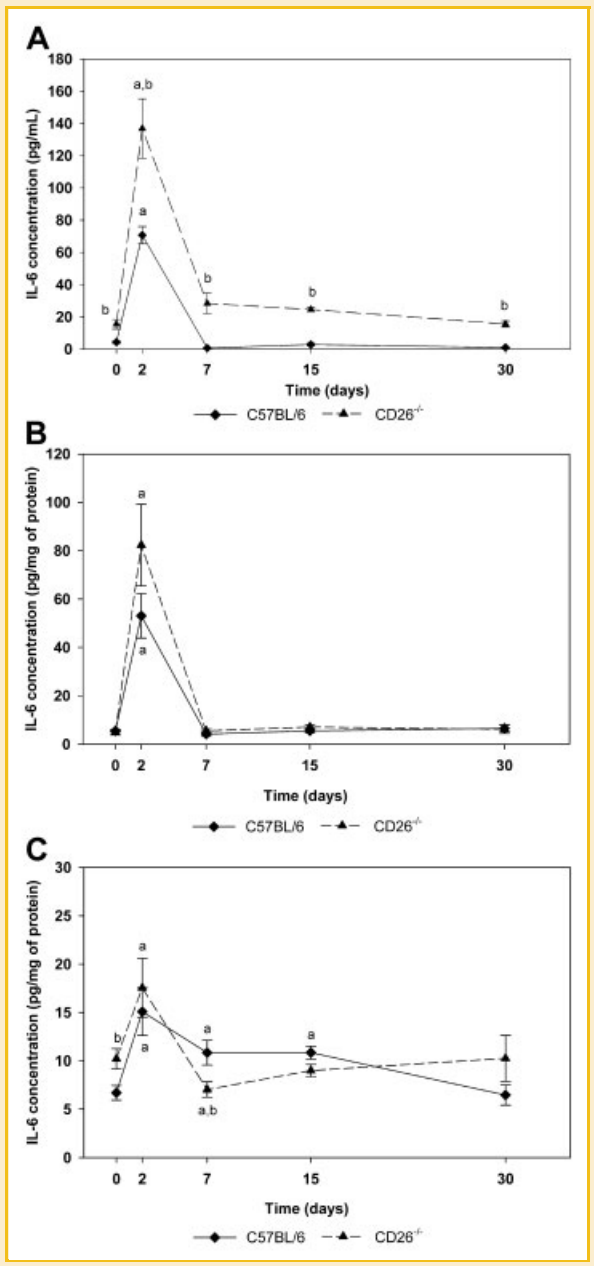


Fig. 7. (A) Serum, (B) colon, and (C) brain IL-6 concentrations at different time schedule during colitis development and resolution in $CD26^{-/-}$ and C57BL/6 mice. [a]Statistically significantly different ($P < 0.05$) from related control group. [b]Statistically significantly difference ($P < 0.05$) between $CD26^{-/-}$ and C57BL/6 mice.

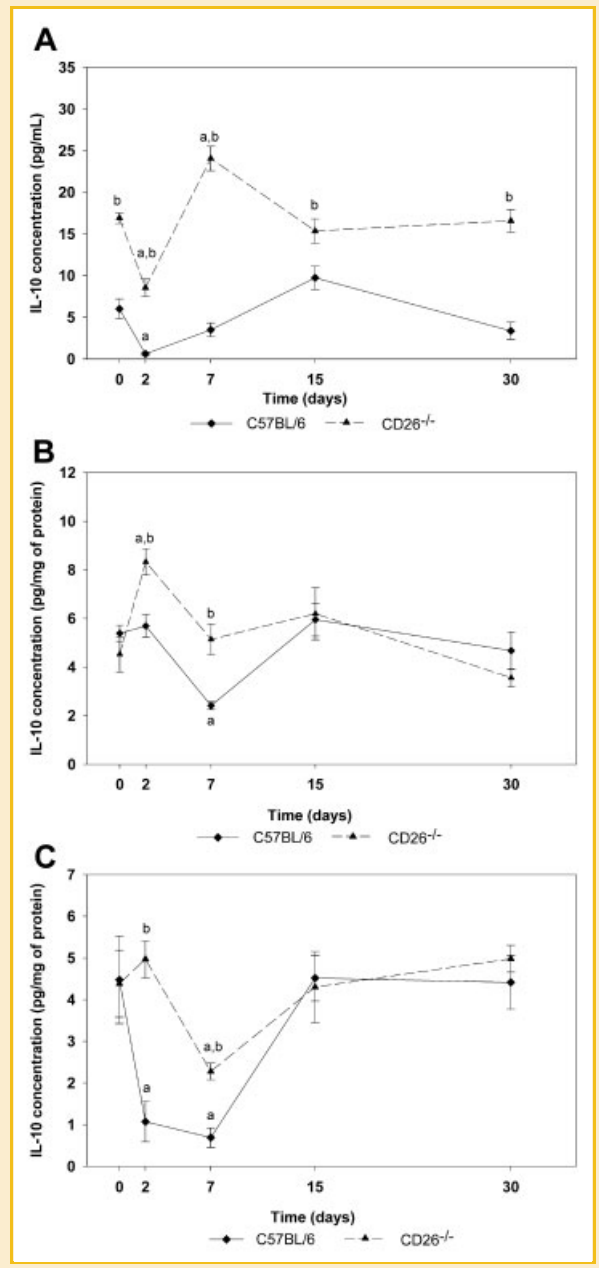


Fig. 8. (A) Serum, (B) colon, and (C) brain IL-10 concentrations at different time schedule during colitis development and resolution in $CD26^{-/-}$ and C57BL/6 mice. [a]Statistically significantly different ($P < 0.05$) from related control group. [b]Statistically significantly difference ($P < 0.05$) between $CD26^{-/-}$ and C57BL/6 mice.

case of IL-6, $CD26^{-/-}$ mice were found to have statistically significantly higher ($P < 0.05$) serum IL-10 values, compared to C57BL/6 mice (Fig. 8A). IL-10 concentrations decrease in both mice strains in acute phase of colitis. Seven days after administration of TNBS solution, serum IL-10 concentrations significantly increase in $CD26^{-/-}$ mice, reaching statistically significantly higher values ($P < 0.05$) compared to physiological conditions. On the other hand, C57BL/6 mice had a statistically significantly ($P < 0.05$) increased serum IL-10 concentration 15 days after colitis induction, in later days of colitis resolution, when $CD26^{-/-}$ mice have already achieved physiological values.

Results of our study showed that $CD26^{-/-}$ and C57BL/6 mice do not differ statistically significantly in the content of colon IL-10 in physiological conditions (Fig. 8B). Under conditions of colon inflammation, statistically significant differences were found between investigated mice strains. CD26 deficiency caused a statistically significant increase in colon IL-10 concentration in the acute phase of inflammation, compared to physiological

conditions. On the other hand, C57BL/6 mice did not show significant changes in the acute phase. Furthermore, a statistically significant decrease in the content of IL-10 in the colon was found the 7th day after colitis induction in C57BL/6 mice, compared to their controls. Generally, under conditions of CD26 deficiency, IL-10 concentrations in colon were statistically significantly higher ($P < 0.05$) in acute phase of inflammation and at the beginning of tissue healing processes, compared to C57BL/6 mice with induced colitis.

Moreover, a sharp decrease in brain IL-10 concentration was observed in C57BL/6 mice, in the acute phase of colitis and when the process of healing starts, while in $CD26^{-/-}$ mice this decrease was less expressed and determined only the 7th day of colitis (Fig. 8C).

## DISCUSSION

Nowadays, the largely accepted fact that IBD is an immune-mediated chronic disorder is enriched with increasing evidence showing that neurogenic inflammation is a key contributing factor in inflammatory mechanisms [Engel et al., 2011]. Results of this study provide new data regarding alterations of significant neuropeptides and interleukins during colitis development under conditions of CD26 deficiency. Obtained results showed that inflammatory changes at the local site of inflammation, in the colon, reflected on investigated parameters in the central nervous system, which suggests and confirms an important role of the gut–brain axis in IBD pathogenesis.

Previous results from different research groups, including ours, provided knowledge regarding causal connection between patients affected with IBD and DPP IV/CD26 [Hildebrandt et al., 2001; Varljen et al., 2005]. It has been shown that inhibitors of DPP IV/CD26 partially ameliorate inflammatory processes and modify the course of disease in a model of IBD in mice [Yazbeck et al., 2008]. Moreover, DPP IV/CD26 inhibitors have recently been proposed as an emerging drug class for treatment of inflammatory diseases [Yazbeck et al., 2009].

Given the potential role of DPP IV/CD26 and the importance of its biologically active substrates in the pathogenesis of IBD, we hypothesized that DPP IV/CD26 could have an important neuroimmunomodulative role at systemic and local levels in processes leading to inflammation and tissue healing in a model of IBD in mice. There is evidence that gastrointestinal inflammation is related to an imbalance in the function of peptidergic neurons, including VIP and NPY [Okajima and Harada, 2006; Chandrasekharan et al., 2008]. VIP has previously been shown to possess anti-inflammatory properties given that treatment with VIP led to the recovery of clinical symptoms, amelioration of parameters of inflammation related to the recruitment and traffic of cell populations in a TNBS-induced model of colitis [Arranz et al., 2008]. Therefore, VIP has been proposed as a potential healing mediator in CD. Our results showed that VIP concentrations increase statistically significantly ($P < 0.05$) in acute inflammation in serum, colon, and brain of both $CD26^{-/-}$ and C57BL/6 mice. Since changes were more conspicuous in $CD26^{-/-}$ mice, it can be concluded that, in the absence of DPP IV/CD26, concentrations of this protective neuropeptide are higher both in physiological conditions and during inflammatory events in experimental colitis at systemic and local levels. Being a DPP IV/CD26 substrate, VIP is truncated via its enzymatic activity in serum and tissues. Our results showed that CD26 deficiency has an important impact on systemic and local VIP concentrations, suggesting a more significant role of DPP IV over DPP IV-like enzymes in its metabolism.

However, although VIP is a known *in vivo* substrate of DPP IV/CD26, it is not entirely clear if increased levels of VIP are in total or partially attributable to its less truncation. It has previously been shown that VIP is upregulated after neural injury and has potent neuroprotective properties [Gressens et al., 1997]. Therefore, another possible explanation of the results of this study could also be that higher concentrations of VIP are produced through an indirect mechanism under inflammatory conditions.

Results of previous research revealed a local VIP augmentation upon DPP IV/CD26 inhibition in experimental lung transplantation. Inhibition of intragraft DPP IV/CD26 enzymatic activity induced preservation of endogenous intragraft VIP levels which correlated with maintaining lung function and structural integrity [Jungraithmayr et al., 2009]. Therefore, application of DPP IV/CD26 inhibitors could have multiple implications in therapeutic approaches to immunologically mediated diseases.

Since DPP IV/CD26 inhibitors are in clinical use for therapy of Diabetes mellitus type 2, it would be worth to know the impact of such inhibition on the neuroimmune response, given its involvement in a wide spectrum of biological functions. Scarce reports are available regarding influence of DPP IV/CD26 inhibition on biological activity and actions of neuropeptides and other biogically active compounds. Therefore, investigations including patients under therapy with DPP IV/CD26 inhibitors and their influence on the neuroimmune response should be performed in order to evaluate possible side-effects of such therapeutic approach.

A potential increase in circulating NPY as a consequence of reduced DPP IV/CD26 activity may have impacts on blood pressure. Jackson and Mi [2008] found that inhibition of DPP IV/CD26 enhanced the ability of exogenous NPY to improve renovascular responses to angiotensin II in kidneys of genetically hypertensive rats. Nevertheless, no complications with blood pressure have been observed in clinical trials investigating the effects of DPP IV/CD26 inhibitors in nondiabetic patients [Mistry et al., 2008]. Furthermore, DPP IV/CD26 inhibition appears to enhance the antilipolytic action of NPY in human adipose tissue [Kos et al., 2009]. Future studies are required to analyze whether this observation could explain the lack of weight loss in diabetic patients treated with DPP IV/CD26 inhibitors.

Results of our study showed a significant impact of DPP IV/CD26 on the regulation of NPY concentrations in serum and tissues. DPP IV/CD26 induced opposite effects on NPY concentration in brain in analyzed mice strains, which could not be attributed only to its proteolytic activity, but most probably it is a consequence of DPP IV/CD26 costimulatory actions on immune cells and involvement in T lymphocyte activation [Reinhold et al., 2009]. Activated T lymphocytes express high levels of DPP IV/CD26 and specific DPP IV/CD26 inhibitors have been shown to suppress cytokine production of stimulated human and murine T lymphocytes

[Matteucci and Giampietro, 2009]. A causal connection in these processes is not well understood, but it is known that NPY is readily processed by DPP IV/CD26, affecting its capability to bind to its receptor NPY-Y1 [Mentlein, 1999]. Murine T lymphocytes also express functional NPY-Y1 receptors [Gehlert, 2004]. It has previously been shown that experimental colitis is attenuated by preventing NPY-Y1 signaling [Hassani et al., 2005]. Furthermore, targeted deletion of NPY was confirmed to modulate experimental colitis and greater susceptibility to colitis development was found in C57BL/6 mice compared to NPY-deficient mice [Chandrasekharan et al., 2008].

Given the fact that our results showed statistically significantly higher ($P < 0.05$) NPY concentrations in colon of C57BL/6 mice compared to CD26$^{-/-}$ in both acute phase of inflammation and at time when tissue healing takes part, a potential protective role of DPP IV/CD26 deficiency could be implied in those processes. Our results of NPY concentrations in brain additionally confirmed the relevance of a causal connection between DPP IV/CD26 and NPY during inflammatory events, showing opposite changes under conditions of DPP IV/CD26 deficiency. Therefore, it can be concluded that DPP IV/CD26, via both its proteolytic function and involvement in complex immunological mechanisms like T-cell activation, cytokine secretion, and immunoglubulins production [Fan et al., 2003], is implicated in neuroimmunological processes, including modulation of VIP and NPY concentrations at systemic and local levels.

Acute inflammation of the colon in our studied model was characterized by an enhanced concentration of IL-6 in serum of both CD26$^{-/-}$ and C57BL/6 mice. Likewise, concentrations of IL-6 were increased at the site of inflammation in both mice strains, additionally confirming the presence of inflammatory processes in the colon. Our results are in agreement with previously published data regarding patients affected with CD, showing high levels of IL-6 concentration in both serum and intestinal tissues [Gross et al., 1992; Holtta et al., 2008]. As in other investigated parameters in this study, inflammatory events in the colon reflected on alterations of IL-6 concentrations in the brain.

Earlier experiments have proved that IL-6 plays an important role in the course of colitis [Ding et al., 2009]. Furthermore, IL-6-deficient mice were found to be partially protected from TNBS-induced colitis in mice, most likely via their significantly elevated baseline levels of anti-inflammatory cytokines [Gay et al., 2006]. CD26$^{-/-}$ mice constitutionally showed higher IL-6 serum and brain concentrations while no differences were found in colon IL-6 concentration compared to C57BL/6 mice. Our results are in agreement with previously published studies regarding secretion of cytokines and immunoglobulins in CD26$^{-/-}$ mice after activation of immune response [Yan et al., 2003]. Nevertheless, in spite of an accentuated and expected increase of IL-6 in inflamed colon, no statistically significant differences were found between CD26$^{-/-}$ and C57BL/6 mice. Therefore, results from our and other study groups showed that even though IL-6 is a potential substrate of DPP IV/CD26 [Hildebrandt et al., 2000], CD26$^{-/-}$ mice are not more vulnerable to IL-6 actions compared to C57BL/6 mice. Most probably, on one hand because other proteases showing similar DPP IV/CD26 activity undertake its action, and on the other hand because of interactions with other biologically active molecules, such as VIP and IL-10, as demonstrated by our study.

The importance of IL-10 in IBD pathogenesis is supported by evidence that IL-10-deficient mice spontaneously develop colitis [Rennick and Fort, 2000]. Furthermore, IL-10 gene therapy was found to prevent inflammatory changes in TNBS-induced colitis model [Lindsay et al., 2002]. Results of this study indicated that CD26$^{-/-}$ mice have constitutionally statistically significantly ($P < 0.05$) higher IL-10 concentrations in serum compared to C57BL/6 mice, while no differences were found in colon and brain. In the colitis model used in this study, IL-10 concentrations in serum were found to be decreased in the acute phase of colitis in both mice strains. However, CD26$^{-/-}$ mice showed a prompt increase in IL-10 concentration the 7th day after induction of colitis, after which its concentration normalizes to physiological conditions. On the other hand, C57BL/6 mice took longer time to undertake similar pattern of changes. This prompt increase in IL-10 concentration in CD26$^{-/-}$ mice could be explained as a consequence of DPP IV/CD26 deficiency, since IL-10 is also a potential DPP IV/CD26 substrate. Furthermore, DPP IV/CD26 deficiency significantly alleviated changes of IL-10 concentration in the brain, additionally showing an important role of DPP IV/CD26 in immunomodulatory processes. Therefore, our results suggest a potential significant role of DPP IV/CD26 in normalizing IL-10 concentrations in serum and tissues in inflammation. Results of this study are in agreement with previously published research including patients affected with IBD, showing that serum IL-10 is increased during disease recovery [Mitsuyama et al., 2006].

Taken all together, results of this study indicate that DPP IV/CD26 possess neuroimmunomodulative properties in a TNBS-induced colitis in mice. Although both mice strains developed colitis, results of biochemical determinations at molecular level showed a different pattern of changes in concentrations of investigated neuropeptides and interleukins in CD26$^{-/-}$ mice. Besides the fact that other DPP IV/CD26-like peptidases could undertake its action in CD26$^{-/-}$ mice, statistically significant differences in analyzed parameters were found in comparison to C57BL/6 mice, indicating an important role of DPP IV/CD26. Therefore, a partially protective role of DPP IV/CD26 deficiency in inflammatory events, supported by previously published results that showed positive effects of DPP IV/CD26 inhibitors in ameliorating the severity of inflammation in animal IBD models [Yazbeck et al., 2008, 2009], could be proposed.

It has previously been shown that inhibiting DPP IV/CD26 may cause changes in chemokine regulation and consequent anti-inflammatory effects [Yazbeck et al., 2009, 2010]. Furthermore, *in vitro* studies showed that inhibition DPP IV/CD26 activity can induce a decreased secretion of proinflammatory cytokines, such as TNF-α and IFN-γ, as well as an increase in anti-inflammatory cytokines like TGF-β [Reinhold et al., 1997]. Moreover, GLP-2, a DPP IV/CD26 substrate involved in the maintenance of mucosal tissue integrity and tissue healing, was proposed as potential IBD therapeutic agent. DPP IV/CD26 inhibition markedly enhances GLP-2 intestinotrophic properties in both rats and mice [Hartmann et al., 2000]. However, animal studies showed that inhibitors are protective only when DPP IV/CD26 is present, due to protective

conformational change driven events of DPP IV/CD26 inhibitors [Yazbeck et al., 2010].

In conclusion, results of this study provide new data and for the first time, as far as we are aware, give insights into alterations of investigated neuropeptides and interleukins at systemic and local levels during colitis development and tissue healing in TNBS-induced colitis in CD26$^{-/-}$ mice. DPP IV/CD26 deficiency was found to affect the neuroimmune response during colitis development and resolution in mice. Inflammatory changes at the local level, in the colon, reflected on investigated parameters in the brain, suggesting an important role of the gut–brain axis in IBD pathogenesis.

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