Running title: Role of BDNF in intestinal barrier in mice

Brain-derived neurotrophic factor modulates intestinal barrier by inhibiting intestinal epithelial cells apoptosis in mice

Dong-Yan Zhao[1], Wen-Xue Zhang[2],Qing-Qing Qi[2], Xin Long[2], Xia Li[2],Yan-Bo Yu[2*], Xiu-Li Zuo[2]

[*]**Correspondence:**

Yan-Bo Yu, M.D.Ph.D.

E-mail: yuyanbo2000@126.com

Tel: +86-531-82166012; Fax: +86-531-82166012

[1] Department of Gastroenterology, General Hospital of Puyang Oilfield, Puyang 457000, P. R. China

[2]Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan 250012, P.R. China

Abstract

Background We aimed to investigate the effects of brain-derived neurotrophic factor (BDNF) on apoptosis of intestinal epithelial cells (IECs) and alterations of intestinal barrier integrity using BDNF knock-out mice model.

Methods Colonic tissues from $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice were prepared for this study. The integrity of colonic mucosa was evaluated by measuring trans-mucosa electrical resistance and tissue conductance in Ussing chamber. The colonic epithelial structure was analyzed by transmission electron microscopy. Apoptosis involvement was determined with TUNEL staining, active caspase-3 immunostaining and Western blotting for the protein expression of active caspase-3, Bax and Bcl-2. The expression levels and distribution of tight junction proteins were evaluated by immunohistochemistry or Western blots.

Results Compared with $BDNF^{+/+}$ mice, $BDNF^{+/-}$ mice displayed impaired integrity and ultrastructure alterations in their colonic mucosa, which was characterized by diminished microvilli, mitochondrial swelling and epithelial cells apoptosis. Altered intestinal barrier function was linked to excessive apoptosis of IECs demonstrated by the higher proportion of TUNEL-positive apoptotic cells and enhanced caspase activities in $BDNF^{+/-}$ mice. Increased expression of Bax and claudin-2 proteins and reduced Bcl-2and tight junction proteins (occludin, ZO-1 and claudin-1) expression were also detected in the colonic mucosa of $BDNF^{+/-}$ mice.

Conclusions BDNF may play a role in the maintenance of intestinal barrier integrity

via its anti-apoptotic properties.

Key words

BDNF – intestinal barrier – intestinal epithelial cells – apoptosis

Introduction

Intestinal epithelial cells (IECs) play an important role in the maintenance of intestinal homeostasis. IECs provide a physiological barrier that prevents the entry of harmful antigens, microbes and allows nutrients and water transport from the intestinal lumen to the blood. Loss of epithelial integrity has been implicated as a critical factor in the predisposition to a number of gastrointestinal diseases, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and celiac disease (Liu *et al.* 2011, Piche *et al.* 2009, Rauhavirta *et al.* 2014). Multiple mechanisms are responsible for maintaining the integrity of epithelial barrier (Jeon *et al.* 2013). Of note, growing evidence has demonstrated the important role of epithelial cell death by apoptosis in the regulation of barrier function (Sun *et al.* 1998, Zhang *et al.* 2002)

The balance between cell apoptosis and regeneration plays a critical role in the maintenance of intestinal integrity. Under physiological conditions, new epithelial cells are derived from stem cells in the crypt and then differentiate into mature forms to replace apoptotic cells shedding from the surface epithelial cuff (Gunther *et al.* 2013, Potten and Loeffler 1990). Spontaneous or induced excessive apoptosis of IECs can result in disruption of epithelial integrity and permeability defects during inflammatory, diet and infection-induced disease processes (Gunther *et al.* 2013). For

example, patients suffering from IBD showed increased numbers of apoptotic IECs with corresponding barrier defects and the consequent translocation of bacteria into the intestinal wall (Maloy and Powrie 2011).

Preserving the integrity of the epithelial barrier by regulating the rate of cell death is considered crucial for maintaining intestinal homeostasis. A complex network of regulatory peptides, such as cyclooxygenase-2 (Fredenburgh *et al.* 2011), trefoil factor (Hanisch *et al.* 2017), glucocorticoids (Amsterdam *et al.* 2002) and transforming growth factor (TGF)-β (Dignass and Podolsky 1993), have been identified as strong cytoprotective factors; thus, this factors play an important role in inhibiting apoptosis of IECs and mucosal defense. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is originally known to play an important role in controlling cell survival, differentiation and death in the nervous system (Arevalo and Wu 2006). An *in vitro* study indicated that the addition of BDNF could attenuate apoptosis induced by TNF-α and IFN-γ in enteric glial cells (EGCs) (Steinkamp *et al.* 2012). In human vascular endothelial cells, BDNF maintains the integrity of blood vessel walls and avoids vascular leakage by protecting cells from apoptosis in response to inflammatory mediators (Matsuda *et al.* 2015, Takeda *et al.* 2013). In addition, BDNF is highly expressed in colorectal cancer compared with non-tumor tissues, and increased expression of BDNF can inhibit spontaneous cell apoptosis in tumorigenesis (Brunetto de Farias *et al.* 2010). These findings are further supported by another study in which genetic knock-out of BDNF in cultured colonic cells was associated with an increased rate of apoptosis and a decreased rate of cell growth (Yang *et al.* 2013).

These studies raise the possibility that BDNF may play a vital role in the regulation of enterocyte apoptosis. As is known, BDNF has been confirmed expressed in the gut mucosa of various species, including humans and mice (Chen *et al.* 2012, Yu *et al.* 2012). It has been demonstrated that immunoreactions to BDNF are highly up-regulated in colonic biopsies taken from patients suffering from Crohn's disease and ulcerative colitis (Johansson *et al.* 2007, Steinkamp *et al.* 2012). Besides, massive apoptosis of colonocytes have been demonstrated in patients suffering from IBD, which could cause the focal disruption of epithelial integrity (Di Sabatino *et al.* 2003, Gitter *et al.* 2001). However, whether BDNF is involved in the regulation of epithelial barrier function by affecting apoptosis of intestinal epithelial cells remains unknown.

The purpose of the present study was to investigate the effects of BDNF on apoptosis of IECs and alterations of intestinal barrier integrity using a BDNF knock-out mouse model. We also evaluate possible related molecules involved in BDNF induced apoptosis of IECs. Heterozygous mice ($BDNF^{+/-}$) were used in this study because a previous study demonstrated that homozygous mice ($BDNF^{-/-}$) mice would develop sensory deficits, severe respiratory problems, and die within 3 weeks after birth (Erickson *et al.* 1996).

Methods

Animals

Five heterozygous male $BDNF^{+/-}$ mice and wild-type male $BDNF^{+/+}$ littermates were generous gifts from the Neurobiology Laboratory of Shandong University. The line of $BDNF^{+/-}$ mice generated as described previously was maintained on a C57BL/6

genetic background by backcrossing for 15-18 generations (Ernfors *et al.* 1994). The animals were housed under conditions of controlled temperature (21±1°C), humidity (50±5%) and lighting (12-h day/12-h night). All experiments were performed with adult mice that were 4 months of age. Experimental protocols were approved by the Animal Care and Use Committee of Shandong University, and were conducted in accordance with the guidelines of Chinese Institutional Animal Care Committee.

Tissue processing

After an overnight fast, mice were sacrificed by cervical dislocation. For each mouse, five colonic segments of approximately 1cm in length were prepared. One segment of distal colon was processed for further TUNEL analysis and immunohistochemistry. One segment of distal colon was fixed for electron microscopy observation. Two segments of proximal colon were removed for Ussing chamber experiments. For the final segment of proximal colon, colonic mucosa was obtained by blunt stripping from muscularis and serosa and then snap frozen in liquid nitrogen and stored at –80°C for further Western blotting analyses.

Ussing chamber experiments

Segments of the proximal colon were opened along the mesenteric border. The serosa layer and muscularis layer were carefully removed under an inverted microscope. The murine colon tissue was then mounted in Ussing chambers (Physiologic Instruments, San Diego, CA) with an exposed tissue area of 0.3 cm^2. After mounting, each side of chambers was filled with 5 mL of preheated 37°C Krebs buffer, and continuously oxygenated with 95% O_2 and 5% CO_2. After 15 min of equilibration, voltage changes

were recorded continuously for 60 min followed by intermittent current pulses of 0.001 mA. . Trans-mucosa electrical resistance (TER, $\Omega \cdot cm^2$) and tissue conductance (S/cm^2) were calculated from the spontaneous potential difference and short-circuit current according to Ohm's law by using the software of Acquire and analyze 2.3. The values of TER and tissue conductance are the mean value of the two segments, with a calculated individual coefficient of variability < 5%.

Transmission electron microscopy (TEM)

Colonic segments for TEM were immediately fixed in a cacodylate-buffered 2.5% glutaraldehyde solution at 4°C overnight, washed in cacodylate buffer (0.1 mol/L), and postfixed with 1% osmiumtetroxide for 1 h at room temperature. After washing in cacodylate buffer (0.1 mol/L), the samples were dehydrated in graded concentrations of acetone, and embedded in Araldite. Samples were cut into semithin sections (500 nm) with an ultramicrotome equipped with a glass knife while ultrathin sections (70 nm) were cut on the same microtome equipped with a diamond knife. Toluidine blue-stained semithin sections were screened under an optical microscope to observe colonic epithelial layers. Following this, ultrathin sections were double stained with uranyl acetate and lead citrate, and observed under a JEOL CX1200 electron microscope.

Immunohistochemistry

Colonic segments from $BDNF^{+/-}$ mice and $BDNF^{+/+}$ mice were fixed in 10% neutral buffered formalin. Immunohistological studies were performed on paraffin-embedded 4-μm thick sections. The sections were heated in boiling

Tris-ethylenediaminetetraacetic acid (0.01 M, pH 6.0) for15 min in a microwave oven and endogenous peroxidase activity was blocked by 3% hydrogen peroxide(Rabbit SP kit, Zhongshan Gold Bridge, Beijing, China). Following antigen unmasking, sections were incubated overnight at 4°C with rabbit anti-active-caspase-3 antibody (1:300, Abcam, Cambridge, UK), rabbit anti ZO-1 antibody (1:500, Invitrogen, CA, USA) and rabbit anti-occludin antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then washed in phosphate-buffered saline (PBS) and incubated at room temperature for 2h with anti-rabbit secondary antibody (Rabbit SP kit, Zhongshan Gold Bridge, Beijing, China) according to the manufacturer's instructions. Diaminobenzidine (DAB) was used as the chromogen, followed by counterstained with haematoxylin and viewed under a light microscope (Olympus Bx51). To provide a negative control, the same procedure was performed with the omission of primary or secondary antibodies.

Occludin and ZO-1-immunoreactive areas per square millimeter of mucosa were quantified in at least five representative non-overlapping high power fields (HPFs) at ×400 magnification by using the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, Maryland, USA). For tight junction proteins analysis, the immunostaining was measured by the integrated optical density (IOD) with the above software, and the results were expressed as IOD/sum stained area to indicate the mean intensity of staining, which represented the expression levels of tight junction proteins.

Labelling of apoptotic cells

Apoptotic colonic epithelial cells were labeled in situ by identifying DNA fragmentation on paraffin embedded sections using the terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay (Roche, Mannheim, Germany). The criteria for identifying apoptotic cells included condensation of chromatin, brown-stained nuclei and formation of a surrounding halo. The rate of apoptosis was calculated as the ratio of TUNEL-positive cells to the total number of cells counted. This comprised of formal counting of the number of TUNEL-positive cells present within 5 random high power fields of intestinal epithelial cells (>150 enterocytes/field).

Western blotting analysis

Colonic tissue was homogenized in RIPA buffer containing protease inhibitors (Zhongshan Gold Bridge, Beijing, China). The tissue extract was subject to centrifuge at 12,000 rpm for 20 min at 4°C. Protein concentrations were determined using a BCA Protein Assay kit (Solarbio, Beijing, China). Total protein was added to a loading buffer and boiled for 10 min until fully denatured.

Proteins were separated and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, USA). The membranes were incubated at 4°C overnight with rabbit polyclonal anti-BDNF antibody (1:500, Abcam, Cambridge, UK), rabbit polyclonal anti-active caspase-3 antibody (1:300, Abcam, Cambridge, UK), rabbit monoclonal anti-Bcl-2 antibody (1:1000, Abcam, Cambridge, UK), rabbit monoclonal anti-Bax antibody (1:1000, Abcam, Cambridge, UK), mouse anti-claudin-1 antibody (1:500, Invitrogen, CA, USA) or mouse anti-claudin-2

antibody (1:500, Invitrogen, CA, USA). After washing by Tris-buffered saline containing 0.1% Tween-20 (TBST) 3 times, the membranes were subsequently incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:1000, Zhongshan Gold Bridge, Beijing, China) at room temperature for 1 h. The bands were detected by using the enhanced chemiluminescence technique (Amersham, Buckinghamshire, UK) on Kodak BioMax light film. Chemiluminescent signals of protein bands were quantified by ImageJ compared to β-actin band density (1:10000, Zhongshan Gold Bridge, Beijing, China).

Statistical analysis

All values are expressed as the means±SEM. Statistical analyses were conducted using SPSS 20.0 (SPSS, Chicago, IL, USA). Significance of differences between $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice was evaluated by the two-tailed Student's t-tests. Spearman rank correlations were applied for correlation analyses between TER and colonic apoptotic rate. Data were considered statistically significant when $P< 0.05$.

Results

Colonic mucosal BDNF level

The levels of BDNF in colonic mucosa were significantly reduced in $BDNF^{+/-}$ mice compared with $BDNF^{+/+}$ mice (Fig 1A). And the levels of BDNF were reduced by approximately half in transgenic mice (83.93%±9.74% *vs* 49.23%±7.36%, P=0.031)(Fig 1B).

Colonic mucosal integrity

The integrity of colonic mucosa in $BDNF^{+/-}$ mice was evaluated by measuring TER

and tissue conductance in Ussing chambers. The TER values were significantly lower in $BDNF^{+/-}$ mice than in $BDNF^{+/+}$ mice (141.77±11.13Ω·cm^2 *vs* 82.74±10.60Ω·cm^2, P=0.005), and the conductance was greater in $BDNF^{+/-}$ mice(0.0075±0.0007m/cm^2 *vs* 0.0130±0.0018m/cm^2, P=0.004), suggesting that $BDNF^{+/-}$ mice displayed impaired colonic mucosal integrity (Fig 1C and 1D).

Ultrastructural findings of the colonic epithelium

We performed ultrastructural analyses to further evaluate the mucosal integrity of $BDNF^{+/-}$ mice. The colonic epithelium of $BDNF^{+/+}$ mice consisted of regular columnar cells with numerous apical microvilli on the apical surface of the cell (Fig 2A). The epithelial cells exhibited a central and elongated nucleus featuring prominent euchromatin and a cluster of heterochromatin on the edge (Fig 3A). The electron-dense cytoplasm presented homogeneous content with numerous transparent vesicles and rounded mitochondria with well-developed and observable cristae (Fig 3A and 3C). In $BDNF^{+/-}$ mice, some epithelial cells presented striking changes of their ultrastructure, including diminished or partially disappeared microvilli, less electron-dense cytoplasm (Fig 3D), an increased number of vacuolated vesicles (Fig 2B), swollen mitochondria with disrupted or blurred cristae (Fig 3D). Besides, the nuclei became irregularly condensed, pyknotic and featured a predominant of heterochromatin compared with $BDNF^{+/+}$ mice (Fig 3B). Some vacuoles containing cytoplasmic constituents, cell granules and apoptotic bodies were blebbing from the cytoplasm (Fig 3E). Also, the less electron-dense cytoplasm, apoptotic bodies (Fig 2B) and the condensed nuclei (Fig 3B) indicated apoptosis of colonic epithelial cells in

$BDNF^{+/-}$ mice. In some samples, the apical part of the intercellular junction complex between two adjacent epithelial cells was damaged with widen intercellular space(Fig 2C).

Apoptosis and caspase-3 activation in $BDNF^{+/-}$ mice

TUNEL staining, as well as immunohistochemistry and Western blots for active caspase-3 were carried out for further supporting apoptosis of colonic epithelial cells in $BDNF^{+/-}$ mice. For $BDNF^{+/-}$ mice, TUNEL-positive labeled epithelial cells were scattered in both the cuff and the crypt of the colonic epithelium (Fig 4A). However, no positive staining was found in the crypt of the epithelium in $BDNF^{+/+}$ mice (Fig 4A). By image analysis, the proportion of TUNEL-positive cells was significantly increased in the colonic epithelium of $BDNF^{+/-}$ mice compared with $BDNF^{+/+}$ mice ($9.28\% \pm 0.60\%$ *vs* $34.70\% \pm 1.44\%$, $P<0.001$) (Fig 4A). Besides, a statistical correlation was drawn between the apoptotic rate and TER of colonic segments in $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice ($rs=-0.70$, $P=0.024$). By immunostaining, colonic epithelial cells of $BDNF^{+/-}$ mice exhibited positive immunolabeling for active caspase-3 (Fig 4B). Similarly, Western blot analyses demonstrated that the expression levels of active caspase-3 in colonic epithelium were significantly elevated in the $BDNF^{+/-}$ mice than in $BDNF^{+/+}$ mice ($24.12\% \pm 9.57\%$ *vs* $70.48\% \pm 8.25\%$, $P=0.007$)(Fig 4B).

Apoptosis-related proteins levels in colonic mucosa

The expression levels of apoptosis-related proteins were investigated by Western blot. Compared with $BDNF^{+/+}$ mice, the protein levels of Bcl-2 in the colonic epithelium of

$BDNF^{+/-}$ mice were significantly reduced (70.01%±10.68% *vs* 38.41%±4.91%, P=0.023), while the protein levels of Bax were remarkably increased in $BDNF^{+/-}$ mice (21.53%±7.11% *vs* 70.70%±10.91%, P=0.009)(Fig 5A). Consequently, the ratio of Bcl-2/Bax in the colonic mucosa was significantly decreased in $BDNF^{+/-}$ mice compared with $BDNF^{+/+}$ mice(1.89±0.16*vs*0.99±0.24, P=0.036)

Tight junction protein expression levels in $BDNF^{+/-}$ mice

Occludin- and ZO-1-like immunoreactivity was seen abundantly distributed throughout the mucosa in $BDNF^{+/+}$ mice. Quantification revealed that the expression levels of occludin and ZO-1 in the colonic mucosa were significantly lower in $BDNF^{+/-}$ mice than in controls (occludin, 37.86±4.60 *vs* 22.24±2.18, P=0.015; ZO-1, 39.77±4.57 *vs* 22.28±2.98, P=0.012)(Fig 5B). Claudin-1 protein levels were significantly decreased (143.37%±10.74% *vs* 106.72%±6.48%，P=0.019) whereas claudin-2 protein levels were significantly increased (88.40%±9.76% *vs* 140.72%±12.77%，P=0.012) in the intestinal mucosa of $BDNF^{+/-}$ mice compared to control mice.

Discussion

In the present study, we showed for the first time that BDNF deficiency could contribute to intestinal integrity defects in mice, which was illustrated by ultrastructural examinations in $BDNF^{+/-}$ mice. These BDNF-mediated epithelial integrity alterations were further confirmed to be associated with excessive apoptosis of IECs demonstrated by a higher proportion of TUNEL-positive epithelial cells and increased active caspase-3 expression levels. These findings indicated that BDNF

might play a role in the maintenance of intestinal barrier integrity via its anti-apoptotic properties.

As is known, a single layer of intestinal epithelial cells covers the surface of intestine and provides the first line of barrier against luminal antigens. The structural integrity of the intestine and physiological gut homeostasis are only maintained by the rate of cell apoptosis matching the rate of cell renewal (Wong *et al.* 1999). Thus, apoptosis is a critical step responsible for maintaining the integrity of the intestinal mucosal epithelium. In our study, ultrastructural examinations revealed for the first time that barrier damages existed in the colonic mucosa of $BDNF^{+/-}$ mice, which was demonstrated by diminished membrane microvilli, mitochondrial swelling and increased epithelial cells apoptosis. These data raised the possibility that BDNF might inhibit epithelial cell apoptosis and maintain the integrity of the intestine barrier.

BDNF has been reported to be widely expressed in the nervous system. Earlier studies demonstrated that among neurotrophins, BDNF is predominantly involved in neuron proliferation, survival and differentiation (Huang and Reichardt 2001). BDNF has drawn recent attention because of its critical role in non-nervous system. For example, increased expression levels of BDNF have been reported in non-nervous system tumors, such as colorectal cancer (Tanaka *et al.* 2014), choriocarcinoma (Kawamura *et al.* 2013), myeloma (Chu *et al.* 2013), pancreatic cancer (Sclabas *et al.* 2005) and bladder cancer (Lai *et al.* 2010). Moreover, BDNF has the potential for inhibiting endothelial cells apoptosis and modulating endothelial barrier function in the process of periodontal tissue regeneration (Matsuda *et al.* 2015). Farias et al reported that

BDNF could protect HT29 human colonic cells from death in response to cetuximab, which had inhibitory effects on cell proliferation and survival (Brunetto de Farias *et al.* 2010).

In this study, using genetic knock-out mice, we specifically addressed the role of BDNF on the apoptosis of intestinal epithelial cells *in vivo*. TEM study revealed that epithelial cells in $BDNF^{+/-}$ mice demonstrated cytoplasmic hypervacuolization, nuclear chromatin condensation and apoptotic body formation, which were representative signs for apoptosis. TUNEL staining results showed that $BDNF^{+/-}$ mice exhibited an increased number of apoptotic cells in the colonic mucosa. A significant negative correlation was further drawn between TER measured in Ussing chamber and the apoptosis ratio of the IECs. These data suggested that altered intestinal barrier function in $BDNF^{+/-}$ mice was associated with excessive apoptosis of IECs. It should be noted that TUNEL method not only recognizes apoptosis, but also labels cell death by necrosis (Grasl-Kraupp *et al.* 1995, Otsuki *et al.* 2003). Several cells with characteristics of necroptosis were also observed by TEM. That is, showing extensive vacuole formation, mitochondrial swelling and degenerated organelles. To further identify the type of cell death, we performed immunohistochemistry (IHC) for active caspase-3. Caspase-3 is a ubiquitously distributed key player in the terminal pathway of apoptosis, and its activation has been demonstrated to occur together with the characteristic morphological changes that are the hallmarks of apoptosis (Earnshaw *et al.* 1999). In concordance with TUNEL results, we showed that the expression levels of active caspase-3 in the colonic epithelium were significantly higher in the $BDNF^{+/-}$

mice than in $BDNF^{+/+}$ mice. Moreover, $BDNF^{+/-}$ mice demonstrated a marked down-regulation of tight junction proteins, including occludin, ZO-1 and claudin-1 and a marked up-regulation of claudin-2 in the colonic mucosa, which fitted well with ultrastructural alterations of junction complex found by TEM. It is well known that intestinal barrier function is regulated by IECs and intercellular junction complexes in which tight junction is the major constituent (Balda and Matter 2008, Groschwitz and Hogan 2009). All of these findings indicated that BDNF deficiency might lead to excessive apoptosis of IECs in $BDNF^{+/-}$ mice, resulting in the disruption of epithelial barrier. Similar results were also obtained in our previous study(Yu *et al.* 2017). BDNF knockdown in colonic epithelial cell lines reduced the expression of tight junction proteins, providing clear evidence that BDNF can regulate intestinal barrier integrity *in vitro*. It should be noted that claudin-2 was up-regulated in the colon of $BDNF^{+/-}$ mice. In contrast, our previous study showed that claudin-2 protein were significantly decreased in the HT-29cell line after BDNF knockdown *in vitro* (Yu *et al.* 2017). Occludin, ZO-1 and claudin-1 strengthen the barrier and reduce permeability, whereas claudin-2 has been related to cation-selective channel activity and increased permeability (Amasheh *et al.* 2002, Zhang *et al.* 2013). It is unclear how BDNF regulate the expression of claudin-2 *in vivo*. Further studies should be performed to elucidate the precise mechanisms of their interactions. Although tight junction dysregulation is usually thought of as a downstream consequence of excessive IECs death in the epithelial mucosa, several studies have demonstrated that the tight junctions occludin and ZO-1 have signaling properties that activate the pathway of

programmed cell death (Beeman *et al.* 2012, Beeman *et al.* 2009). It remains unclear whether increased IECs death is a secondary event caused by disrupted tight junctions or whether it is a causative event contributing to tight junction dysregulation in genetic mouse models. This needs to be further evaluated.

To investigate the processes underlying our observations of excessive apoptosis of IECs in the colonic mucosa of $BDNF^{+/-}$ mice, we investigated the expression of apoptosis-related proteins (Bcl-2 and Bax). In normal cells, the pro-survival Bcl-2 proteins keep the integrity of mitochondrial membrane and defend cell from death by inhibiting the activation of the pro-apoptotic Bax proteins. The Bcl-2/Bax ratio plays an important role in determining the fate of cells (Delbridge and Strasser 2015, Hale *et al.* 1996). In our study, the protein levels of Bcl-2 were significantly decreased while the activity of Bax protein were markedly enhanced in the colonic mucosa of $BDNF^{+/-}$ mice compared with control mice. In line with our results, one recent study reported the decreased Bcl-2 expression in the BDNF knocked-down human colonic cells (Yang *et al.* 2013). Besides, a decreased Bcl-2/Bax ratio revealed the impaired ability to protect IECs from apoptosis in $BDNF^{+/-}$ mice. Taken together, our data indicated that BDNF might inhibit apoptosis of IECs by affecting Bcl-2/Bax ratio.

There are several limitations of this study. First, the expressions of BDNF in other structural parts, such as enteric nervous system (ENS), were not evaluated in this study. In addition, the potential role of BDNF in the regulation of intestinal epithelial barrier by activating ENS signaling should be further investigated. Second, we focused on the expression of BDNF in only the proximal colon. However, there is

variation in the expression levels of BDNF in different segments of gastrointestinal tract in mice (Lommatzsch *et al.* 1999). Lastly, fluorescein isothiocyanate (FITC)-dextran assays were not used to examine intestinal permeability in the present study. Other quantitative measurements should be used to examine the expression of occludin and ZO-1.

In summary, $BDNF^{+/-}$ mice were characterized by an increased number of apoptotic IECs with corresponding barrier defects in the colonic mucosa. These observations demonstrate the role of BDNF in the maintenance of intestinal epithelial barrier function via its anti-apoptotic properties. The underlying mechanism through which BDNF inhibits apoptosis may be due to its regulation of the balance between Bcl-2 and Bax. Understanding the detailed modulation of intestinal barrier integrity by BDNF may provide new insights into the management of apoptosis-related intestinal inflammatory diseases.

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Ethics approval and consent to participate

Experimental protocols were approved by the Animal Care and Use Committee of Shandong University, and were conducted in accordance with the guidelines of

Chinese Institutional Animal Care Committee.

Authors' contributions

DYZ performed the experiments, analysed and interpreted data and drafted the manuscript.WXZ, QQQ, XL and XL performed the experiments and interpreted the data.YBY obtained the funding, designed the study and critically revised the manuscript. XLZ analysed data and drafted and reviewed the final version of the manuscript. All authors read and approved the final manuscript.

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Figure legends

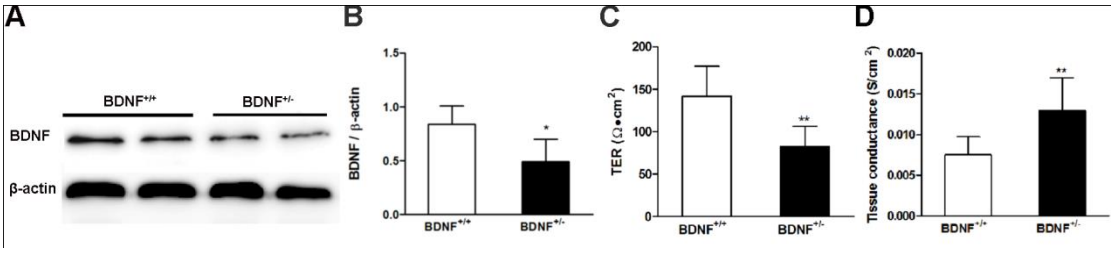

Fig 1 Representative immunoblots (A) and densitometric analyses of BDNF levels (B) in the colonic mucosa of $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice. (C, D) The integrity of colonic mucosa in $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice was evaluated by measuring TER and tissue conductance in Ussing chambers. $^{*}p<0.05$.$^{**}p<0.01$. n=5 per group. TER, trans-mucosa electrical resistance.

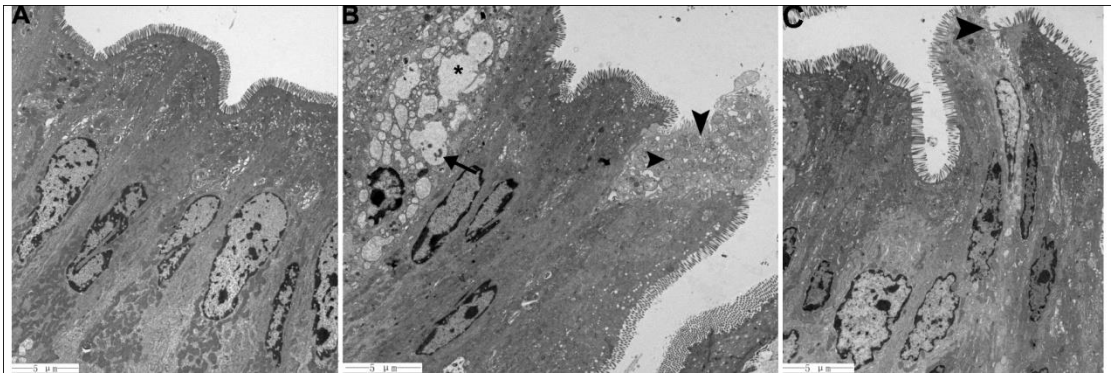

Fig 2 Ultrastructural features of apoptosis in the colonic mucosa of $BDNF^{+/-}$ mice (B). The colonic epithelium of $BDNF^{+/+}$ mice consisted of regular columnar cells with numerous apical microvilli on the plasma membrane (A). The apoptotic cells were readily detected in the colonic mucosa of $BDNF^{+/-}$ mice (B). The ultrastructure of apoptotic cells was characterized by vacuolated cytoplasm (asterisk), apoptotic bodies (arrow) and degenerated cell organelles (arrowhead). (C) The apical part of

intercellular junction complex was partly disrupted(arrowhead).

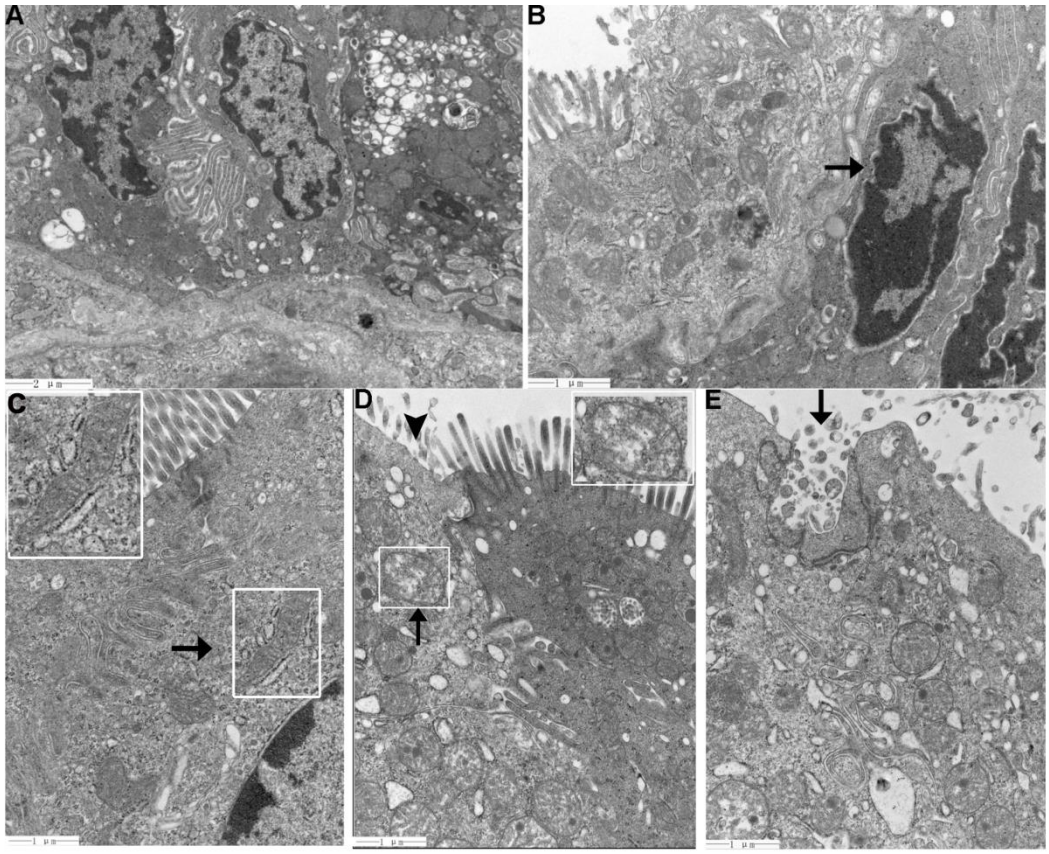

Fig 3 Ultrastructural features of the epithelial cells in $BDNF^{+/+}$ mice (A, C) and $BDNF^{+/-}$ mice (B, D and E). Normal cell nucleus (A) and mitochondria structure (arrow in C) was presented in the epithelium cells of $BDNF^{+/+}$ mice. Damage in $BDNF^{+/-}$ mice was demonstrated by less electron-dense cytoplasm, condensation and margination of nuclear chromatin (arrow in B), swelling mitochondria with blurred cristae (arrow in D) and rarefied microvilli (arrowhead in D). Some vacuoles containing cytoplasmic constituents, cell granules and apoptotic bodies were blebbing from the cytoplasm in $BDNF^{+/-}$ mice (arrow in E).

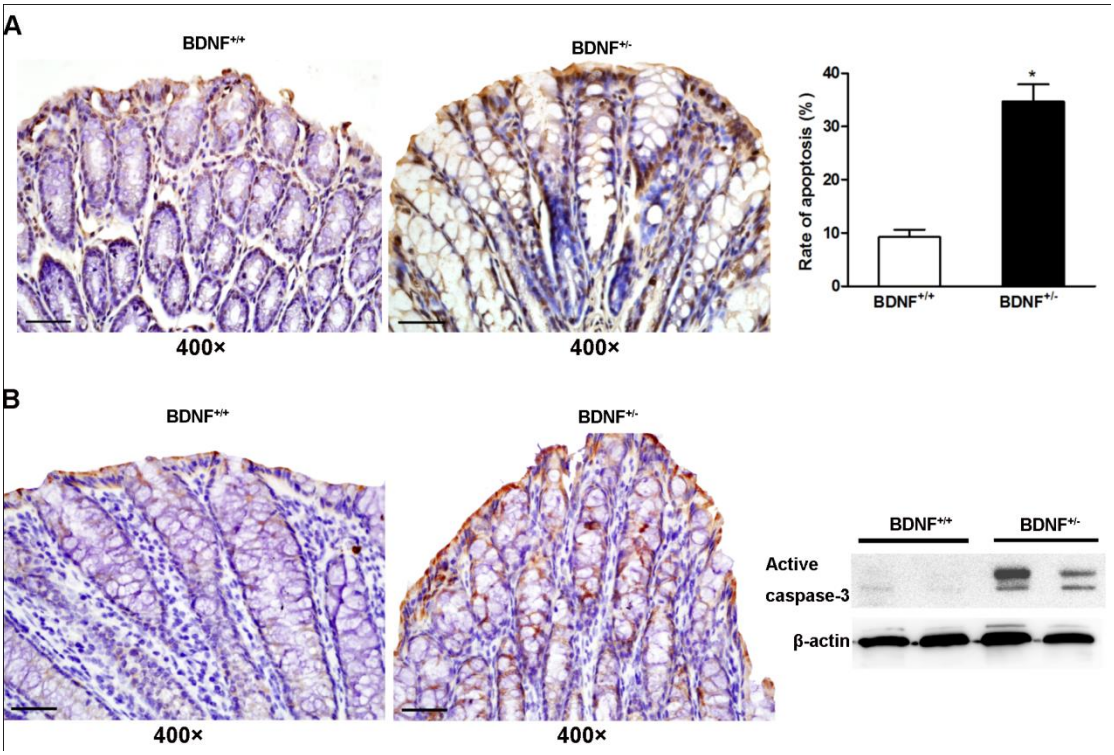

Fig 4 (A) Comparisons of TUNEL staining of the colonic mucosa between $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice. Quantification of TUNEL-positive cells in the colonic mucosa. $^{*}p<0.001$. n=5 per group. Scale bar, 100 μm. (B) Active caspase-3 expression in the colonic mucosa. Representative micrographs showing active caspase-3 immunoreactivity in the colonic section of $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice. Scale bar, 100 μm.

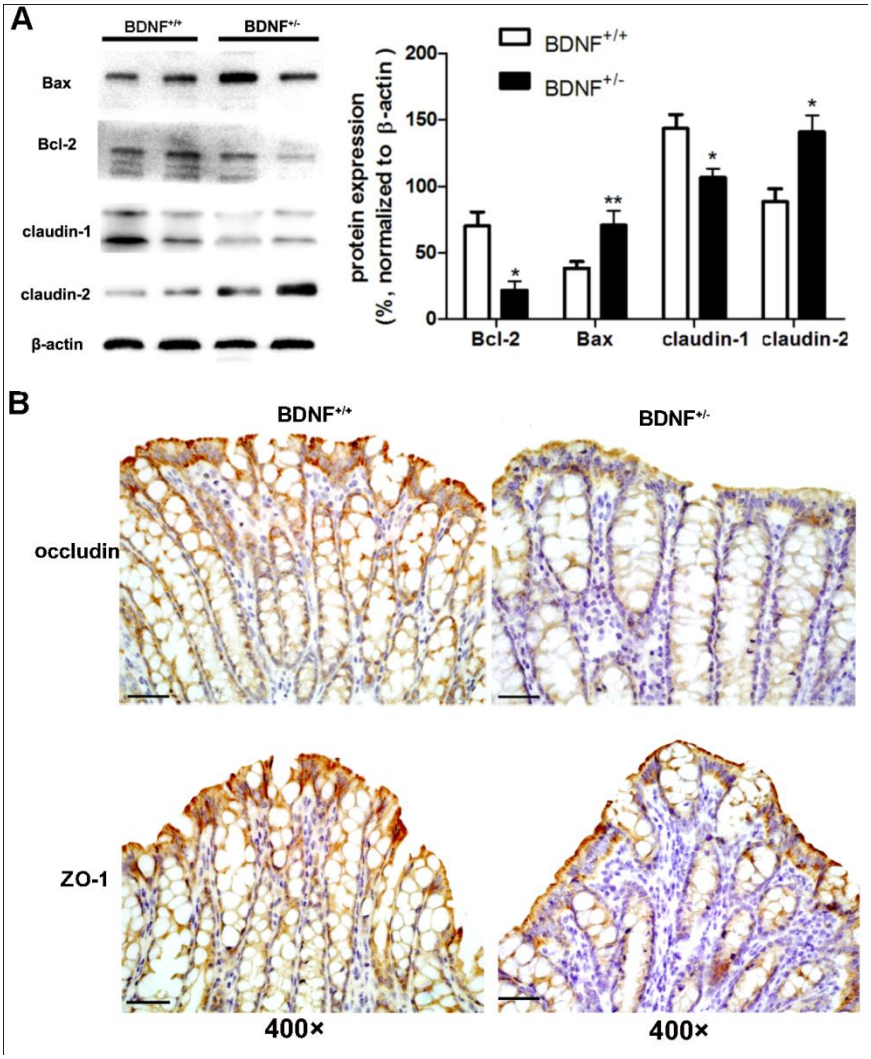

Fig 5 (A) Representative immunoblots and densitometric analyses of Bcl-2, Bax, claudin-1 and claudin-2 in the colonic mucosa of $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice. $^{*}p<0.05$, $^{**}p<0.01$. n=5 per group. (B) Representative micrographs showing occluding and ZO-1 immunoreactivity in the colonic section of $BDNF^{+/+}$ mice and $BDNF^{+/-}$ mice. Scale bar, 100 μm.