Introduction

Diverticular disease (DD) is a widespread disease in industrialized countries with increasing prevalence in the elderly. The initial pathology is characterized by multiple mucosal/submucosal outpouchings throughout the colonic muscle coat which may lead to a broad spectrum of symptoms with potentially lethal complications [1]. Despite the considerably high prevalence, basic research addressing the pathogenesis of DD is lacking behind and the underlying cellular and molecular mechanisms remain largely unknown [2].

Traditionally, low fiber diet, elevated body mass index and connective tissue alterations are considered as risk factors for diverticulitis [3,4]. More recently, novel pathophysiological concepts have addressed alterations of the enteric nervous system (ENS) associated with diverticulitis and the generation of symptoms in chronic DD [5,6]. Previous reports and data from our group have given evidence for an underlying enteric neuropathy in diverticulitis characterized by a decrease of myenteric nerve cells (oligoneuronal hypoganglionosis) and reduced nerve fibers within smooth muscle layers [7,8,9,10]. It is postulated that the disturbed intestinal innervation pattern gives rise to colonic motility disorders frequently reported in DD [11] thereby promoting the development of diverticula. Although a loss of enteric neurons represents a common histopathologic phenotype within the spectrum of gastrointestinal neuromuscular pathology (GINMP) [12], the reason for the reduced ganglionic nerve cell content observed in DD remains unclear.

Survival, differentiation and maintenance of enteric neurons are strongly influenced by neurotrophic factors. Giall cell line-derived neurotrophic factor (GDNF) is a key neurotrophin for the ENS isolated originally from the supernatant of the glial cell line B49 and characterized by its ability to promote the survival of cultured
dopaminergic neurons [13]. GDNF is a member of the TGF-β superfamily of growth factors which regulate numerous functions in the development and differentiation of the nervous system [14]. The GDNF-induced signal transduction is mediated via the glycosyl phosphatidylinositol-anchored receptor GDNF family receptor α1 (GFRα1) and the rearranged during transfection (RET) receptor tyrosine kinase [15]. The impact of the GDNF system on the ENS became evident, when gene-ablated animal models were analyzed for ENS defects. Deletion of GDNF leads to total intestinal aganglionosis, i.e. the complete loss of enteric neurons in the small and large intestine [16]. A similar gross phenotype is observed in mice ablated for the GDNF receptors GFRα1 or RET underlining the essential role of the GDNF system in the development and maintenance of the ENS [17,18].

As GDNF-ablated mice exhibit total intestinal aganglionosis and DD is associated with intestinal hypoganglionosis, we raised the question whether the GDNF system might be compromised in DD thereby contributing to the partial loss of enteric neurons. Therefore, the tissue sources expressing GDNF and its receptors were identified in the human colon and gene expression profiles of the GDNF system were compared between patients with DD and controls. Moreover, the effects of GDNF on GDNF receptor expression as well as on the differentiation and plasticity of enteric neurons were monitored in rat postnatal enteric nerve cell cultures.

Materials and Methods

Patients

Control group. Segments of sigmoid colon were obtained from patients (n = 20, 9 females, 11 males, mean age: 71.3 years) who underwent anterior rectal resection for non-obstructive colorectal carcinoma. Anorectal evacuation and colonic motility disorders as well as the presence of colonic diverticula were previously excluded. Full-thickness specimens were harvested at safe distance (>5 cm) from the tumor and immediately transferred from the operating room to the laboratory for tissue processing. The study of human tissue received approval from the Local Ethics Committee of the Faculty of Medicine, Christian-Albrechts-University of Kiel, Germany (B299/07).

Patients with DD. Segments of sigmoid colon were obtained from patients (n = 21, 14 females, 7 males, mean age: 60.8 years) who underwent sigmoid resection or left hemicolectomy for symptomatic DD. Patients were operated after two or more attacks of diverticulitis by elective surgery. Full-thickness specimens were harvested from sites adjacent to colonic diverticula. Diverticula-containing areas displaying an altered anatomy of the colonic wall due to transmural mucosal/submucosal outpouchings or signs of inflammation and fibrotic scaring were excluded from tissue sampling. The specimens were immediately transferred from the operating room to the laboratory for tissue processing. The study of human tissue received approval from the Local Ethics Committee of the Faculty of Medicine, Christian-Albrechts-University of Kiel, Germany (B299/07).

Tissue Preparation

Tissue processing for mRNA expression profiles of the muscularis propria. The muscularis propria was isolated from full-thickness biopsies of the colonic wall, immediately frozen in isopentane and stored at −70°C until use. Prior to RNA isolation 20 orthogonal cryosections (10 μm) were cut on a cryostat and collected in RNA lysis buffer (Qiagen, Hilden, Germany).

Tissue processing for mRNA expression profiles of LMD samples. Full-thickness biopsies (2 cm border length) were immediately frozen in isopentane and stored at −70°C until use. Orthogonal cryosections (10 μm) were placed on membrane-coated (polyethylene naphthalate, 1.0 μm, Zeiss, Göttingen, Germany) slides. To visualize myenteric ganglia sections were ultra-rapidly (ca. 60 s) stained with toluidine blue and air-dried.

Laser Microdissection and Pressure Catapulting

Laser-microdissection (LMD) was performed by a modified method described previously [19]. Briefly, myenteric ganglia and smooth muscle cells of the muscularis propria were identified by inverse light microscopy (Zeiss Axios Observer Z1, Zeiss, Göttingen, Germany), excised by laser microdissection and collected by laser pressure catapulting (PALM MicroLaser System, Zeiss, Göttingen, Germany) in caps of 0.5 ml reaction tubes. Ganglionic and smooth muscle tissue areas of 2 mm² were collected, immediately dissolved in 350 μl RNA lysis buffer (Qiagen, Hilden, Germany) and stored at −70°C until further use.

Enteric Nerve Cell Culture

Preparation of myenteric ganglionic cells was performed according to a method described previously [20,21]. Briefly, the small intestine was removed from newborn Wistar rats (postnatal day 2–3), the muscularis propria was stripped from the mucosa and placed in Ca²⁺- and Mg²⁺-free Hanks Balanced Salt Solution (HBSS, Gibco Life Technologies, Germany) with antibiotics containing 1 mg/ml collagenase (SIGMA, Munich, Germany). After 2 h of incubation at 37°C fragments of the myenteric plexus were collected under stereomicroscopic control and dissociated by digestion with trypsin/EDTA (0.125 mg/ml Gibco, Life Technologies, Germany) for 15 min at 37°C. The cells were harvested and centrifuged at 900 rpm. Tryptsinization was stopped by replacing it with fetal calf serum (FCS, Gibco, Life Technologies, Germany). The cells were triturated, counted and seeded in a density of 100.000 cells/ml on poly-D-Lysin-(SIGMA)/Laminin-(SIGMA, Munich, Germany) coated coverslips for immunocytochemistry studies or 12-well-plates for gene expression studies. The defined medium for incubating the cells consisted of Neurobasal A (Gibco, Life Technologies, Germany) and B27 supplement (Gibco, Life Technologies, Germany). GDNF (Proprotech, Hamburg, Germany) was added to a final concentration of 2, 10, or 50 ng/ml. For gene expression analysis (GDNF receptors, synaptophysin) and morphometric studies (neuronal counts and network formation) cells were cultured for 1 week, for immunocytochemical detection of synaptophysin culture time was 3 weeks. Medium was changed every second day.

RNA Extraction and Reverse Transcription

Extraction of total RNA from human tissue was performed using a Nucleospin II kit (Macherey and Nagel, Düren, Germany), RNA from enteric nerve cell cultures was isolated using a Nucleospin XS kit (Macherey and Nagel, Düren, Germany) according to the manufacturers guidelines. Prior to reverse transcription, contaminating genomic DNA was digested in a volume of 15 μl using 1.5 U of DNase I (Sigma, Munich, Germany). Reverse transcription was carried out in a total volume of 30 μl containing 200 ng RNA, 375 ng random hexamer primer (GE Healthcare, Freiburg, Germany), 0.5 mM dNTPs (Promega, Mannheim, Germany), 0.01 M DTT, 1× reaction buffer, and 150 U Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). The annealing, elongation, and denaturation steps were carried out at 25°C for 10 min, at 42°C for 50 min, and at 70°C for 15 min, respectively.
Quantitative PCR
Quantitative PCR (qPCR) reactions were run on an ABI Prism 7700 Sequence Detection System (TaqMan, Applied Biosystems, CA, U.S.A.). Amplification reactions were carried out in a 20 μl volume containing 1×qPCR Master Mix Plus (Eurogentec, Cologne, Germany), 900 nM primers, 225 nM hybridization probe, and 2 μl cDNA. Samples were run in duplicate and amplified over 50 cycles. Each cycle consisted of a denaturation phase of 15 s at 95°C and a hybridization/elongation phase of 1 min at 60°C. mRNA expression profiles were measured for GDNF, GFRα1, RET, synaptophysin, and the housekeeping gene HPRT in human samples, and for GFRα2, RET, synaptophysin and HPRT in rat enteric cell cultures. Forward and reverse primers and probes are listed as supporting document Table S1.

Immunocytochemistry of Enteric Nerve Cell Cultures

Immunocytochemistry for morphometric analysis. Cells were fixed for 30 min with 4% paraformaldehyde, permeabilized for 10 min with methanol and treated for 10 min with 3% H2O2. Following blocking of unspecific signals with normal goat serum (1:10) for 30 min, primary antibodies diluted in antibody diluent (Zymed, Invitrogen, CA) were applied for 1 h: mouse anti-HuC/D (1:500, Molecular Probes, Invitrogen) or mouse anti-tubulin βIII (1:200, Merck Millipore, Darmstadt, Germany). HuC/D is a pan-neuronal marker staining specifically neuronal somata thereby allowing reliable identification and counting of nerve cells. Tubulin βIII is a microtubular protein allowing reliable identification of neuronal processes and evaluation of the nervous network. After incubation with the secondary antibody, biotinylated goat anti-mouse IgG (1:200, Jackson Immuno Research, PA) for 30 min and ABC conjugated with horseradish peroxidase were applied for 30 min. DAB (DakoCytomation, Hamburg, Germany) was used as chromogen. Analysis was carried out with a Zeiss Axiophot microscope (Zeiss, Gottingen, Germany).

Dual-label immunocytochemistry. After fixation and permeabilization as described above, samples were incubated with a rabbit anti-synaptophysin antibody (1:1000, Biozol, Echingen, Germany) and a mouse anti-NGF 9.5 antibody (1:1000, Acris, Herford, Germany) for 1 h. After 30 min incubation with the secondary antibodies, goat anti-rabbit-AlexaFlour488 (1:250, Invitrogen, Karlsruhe, Germany) and goat-anti-mouse-Alexafluor546 (1:250, Invitrogen, Karlsruhe, Germany), cells were counterstained with DAPI (Roche, Mannheim, Germany) to visualize cell nuclei. Analysis was carried out with a fluorescent microscope (Axiowert 200M, Zeiss, Gottingen, Germany) coupled to a digital camera (AxioCam, Zeiss, Gottingen, Germany). Image acquisition was performed with Axiovision software (Zeiss, Gottingen, Germany).

Morphometric Analysis of Enteric Nerve Cell Cultures

Technical devices, software. Morphometric analysis was carried out by using a light microscope (Axiophot, Zeiss, Germany) coupled to a digital camera (AxioCam, Zeiss, Germany). The software program Axiovision (version 4.7, Zeiss, Germany) was used for analysis. The data were transferred into Excel software (version 9.0) and further processed for statistical analysis and data plotting (PrizmTM, GraphPad, Sand Diego, CA, USA).

Nerve cell counts. The number of HuC/D-immunoreactive nerve cells was analyzed per optical field (objective with 20× magnification). Neurons were recorded and counted in three randomly assigned optical fields and the mean value was calculated. Experiments were carried out in quadruplicate (50 ng/ml GDNF vs. control).

Neuronal network area. The area occupied by the tubulin βIII-immunoreactive neuronal network was measured and related to the overall-area of the optical field (objective with 20× magnification). Five randomly chosen optical fields were analyzed using an area-analysis-tool (AnalySIS Pro 3.1, Soft Imaging Software, Münster, Germany) to allow reliable depiction of the entire nervous network. The mean value was calculated and expressed as relative area related to the optical field (percentage). Experiments were carried out in triplicate (50 ng/ml GDNF vs. control).

Statistical Analysis
Comparison of mRNA expression levels of the GDNF system between the control group and patients with DD as well as morphometric analysis of enteric nerve cell cultures comparing GDNF-treated cultures vs. controls were carried out by using non-parametric Mann-Whitney U-tests. The mRNA expression of the GDNF system in laser-microdissected myenteric ganglia and muscle layers and the effects of the GDNF treatment on rat enteric nerve cell cultures regarding gene expression studies were analyzed by one-way-ANOVA followed by Newman-Keuls post hoc test (PrizmTM, GraphPad, Sand Diego, CA, USA). Differences were considered significant if p<0.05.

Results

Gene Expression Profiles of the GDNF System in Patients with DD and Controls
To determine the regulation of the GDNF system in patients with DD compared to controls mRNA expression levels of GDNF and its corresponding receptors GFRα1 and RET were monitored by qPCR. Analysis of the muscularis propria revealed that the GDNF mRNA expression was strikingly down-regulated in patients with DD (p<0.001), as mRNA levels dropped to 36% of control values (Fig. 1A). The GDNF receptor GFRα1 was down-regulated to 48% of control values (p<0.001, Fig. 1B) and RET mRNA levels dropped to 29% of mRNA expression detected in controls (p<0.001, Fig. 1C).

Tissue Source of Components of the GDNF System in the Human Colon
To identify the tissue source of components of the GDNF system the intestinal compartments of interest were selectively isolated by LMD. Site-specific gene expression profiles of GDNF and its receptors GFRα1 and RET were monitored in myenteric ganglia as well as in the circular and longitudinal smooth muscle layers. GDNF mRNA levels were significantly higher in the circular (p<0.001) and longitudinal (p<0.05) muscle compared to the myenteric plexus. (Fig. 2A). Highest GDNF expression was found within the circular muscle. GFRα1 was present in all intestinal compartments investigated. Whereas levels in the myenteric plexus and the circular muscle were similar, mRNA expression in the longitudinal muscle exceeded significantly that observed in the myenteric plexus (p<0.001) and circular muscle (p<0.001) (Fig. 2B). RET mRNA expression was significantly higher in the myenteric plexus than in the circular (p<0.001) and longitudinal muscle (p<0.001) (Fig. 2C).

Effects of GDNF on GDNF Receptor Expression in Enteric Nerve Cell Cultures
As we aimed to investigate the putative effect of a reduced GDNF mRNA expression on enteric neurons, we implemented a cell culture model of rat postnatal dissociated myenteric plexus...
exposed to increasing concentrations of GDNF. First, the effect of GDNF on mRNA expression of its corresponding receptors GFRα1 and RET was assessed. After 1 week of GDNF treatment, both receptors showed up-regulation of their respective mRNA expression (Fig. 3). A dose of 50 ng/ml GDNF increased GFRα1 mRNA levels 2.6 fold compared to controls (Fig. 3A), and RET mRNA expression was up-regulated 7.0 fold compared to untreated cell cultures (Fig. 3B). These findings indicate that the growth factor GDNF is able to induce mRNA expression of its receptors responsible for mediating neurotrophic effects.

Effects of GDNF on Neuronal Differentiation in Enteric Nerve Cell Cultures

The neurotrophic capacity of GDNF was studied by assessing the neuronal number and differentiation of the neuronal network in enteric nerve cell cultures exposed to GDNF. The rationale of these experiments was to conclude whether a lack of GDNF may contribute to the relative loss of enteric nerve cells observed in patients with DD. The numerical increase of cultured myenteric neurons was monitored by anti-HuC/D immunocytochemistry. After a culture time of 1 week, few dispersed HuC/D immunoreactive nerve cells were observed in control cultures (Fig. 4A). At the same time, neuronal numbers were significantly (p<0.01) elevated following treatment with 50 ng/ml GDNF (Fig. 4B). Morphometric analysis revealed that GDNF treatment led to a 2.2 fold increase of nerve cells compared to controls (Fig. 4C).

Differentiation of the neuronal network was monitored by anti-tubulin βIII immunocytochemistry. After a culture time of 1 week, control cultures displayed only small-sized neuronal aggregates with few and thin interconnecting nerve fiber strands (Fig. 4D). In contrast, following GDNF treatment neuronal aggregates were larger and the interconnecting nervous network displayed densely ramified nerve fiber strands of markedly increased thickness (Fig. 4E). Morphometric analysis revealed that GDNF treatment led to a 3.6 fold increase (p<0.01) of the neuronal network area compared to controls (Fig. 4F).

Effects of GDNF on Neuronal Plasticity in Enteric Nerve Cell Cultures

The positive effect of GDNF on neuronal differentiation of enteric nerve cell cultures was further addressed by assessing the influence of this neurotrophic factor on neuronal plasticity. Therefore, the effects of GDNF on the synaptic vesicle marker synaptophysin were studied in enteric nerve cell cultures. mRNA expression of synaptophysin was dose-dependently increased following GDNF treatment after one week of culture time (Fig. 5). At a dose of 50 ng/ml, GDNF augmented mRNA levels of the synaptic vesicle marker 10.5 fold (p<0.01) compared to controls. To monitor the topographical expression pattern of synaptophysin, enteric nerve cell cultures were analyzed after 3 weeks of GDNF treatment by dual label immunocytochemistry for synaptophysin and the pan-neuronal marker PGP 9.5 (Fig. 6). Synaptophysin immunoreactivity was detectable both in control
cultures (Fig. 6A) and in GDNF treated cultures (Fig. 6D). However, whereas under control conditions the protein was confined to neuronal somata (Fig. 6B–C), GDNF treatment led to immunoreactive signals present in neuronal cell bodies as well as in neuronal processes (Fig. 6E–F). The nerve fiber strands displayed a punctuate and granular synaptophysin immunoreactivity most likely resembling nerve fiber varicosities with accumulated synaptic vesicles. The findings indicate that GDNF positively influences synaptic vesicle formation thereby promoting neuronal plasticity.

Figure 3. mRNA expression of GDNF receptors in enteric nerve cell cultures following GDNF treatment. Treatment with GDNF for 1 week increases mRNA expression of GDNF receptors GFRα1 (A) and RET (B) in enteric nerve cell cultures. mRNA expression levels are normalized to mRNA expression of the house-keeping gene HPRT. Data are shown as mean +/- SEM, n=14–15 per experimental group, *p<0.05 vs. control. doi:10.1371/journal.pone.0066290.g003

Figure 4. Effects of GDNF on neuronal number and differentiation of enteric nerve cell cultures. Rat enteric nerve cells were cultured for 1 week without (A, D) or with 50 ng/ml GDNF (B, E). Immunocytochemistry for the neuronal marker HuC/D (A, B) shows that neuronal aggregates are more numerous and larger after GDNF treatment. The morphometric analysis (C) confirms the increased neuronal number following GDNF treatment. Immunocytochemistry for tubulin βIII (D, E) visualizes neuronal processes which are more densely distributed and ramified after GDNF treatment. The morphometric analysis (F) confirms the increased area of the neuronal network following GDNF treatment. Magnification 4×. Data are shown as mean +/- SEM, n (HuC/D immunocytochemistry) = 11–12, n (βIII tubulin immunocytochemistry) = 7–8 per experimental group, *p<0.05 vs. control. doi:10.1371/journal.pone.0066290.g004
Discussion

To our knowledge, this is the first study addressing neurotrophic factor deficits in patients with DD and investigating in vitro the underlying cellular and molecular mechanisms. The study reveals four important findings: (1) mRNA expression of GDNF and its receptors GFRα1 and RET is down-regulated in the muscularis propria of patients with DD; (2) the main source of GDNF is the intestinal smooth muscle, while RET is mainly expressed in enteric ganglia in which GFRα1 is also found; (3) GDNF up-regulates its corresponding receptors in enteric nerve cell cultures; (4) GDNF treatment promotes neuronal differentiation and plasticity of enteric nerve cell cultures.

Down-regulation of GDNF in Diverticular Disease

DD is a gastrointestinal disorder with a high prevalence but a still poorly understood pathogenesis. Traditional pathogenetical concepts have advocated over decades the impact of a low-fiber diet as major risk factor for DD. Whereas Painter and Burkitt [22] claimed already 40 years ago that a Western diet low in fibres is positively correlated with the risk of developing DD, a recently published study by Peery et al. [23] showed that high, not low, fiber intake is associated with an increased risk of diverticulosis. These novel findings might lead to a re-addressing of putative factors involved in the pathogenesis of DD. In fact, increasing evidence is given that DD is associated with an enteric neuropathy [5,6]. In line with this concept, we and others recently demonstrated that colonic specimens from patients with DD are characterized by an oligoneuronal hypoganglionosis, i.e. a loss of neurons in the ENS [7,9,10].

Since GDNF- and GDNF receptor-knock-out mice exhibit total intestinal aganglionosis demonstrating the crucial role of this growth factor for the development and maintenance of the ENS [16,17,18], we addressed the question whether the GDNF system might be compromised in a condition associated with enteric neuronal loss such as in DD. Indeed, expression levels of both GDNF and GDNF receptors were significantly down-regulated in...
Expression of GDNF on its Corresponding Receptor System in the Human Colon

Site-specific Gene Expression Profiles of the GDNF System in the Human Colon

To investigate the cellular sources of the components of the GDNF system in the human colon, we applied LMD to those tissue compartments of interest followed by qPCR. The intestinal muscle layers could be identified as the main source of GDNF in the adult human colon, whereas RET was mainly expressed by enteric ganglia. Gfrα1 mRNA expression exhibited highest levels in the longitudinal muscle but was also expressed in enteric ganglia. Thus, the so called “neurotrophic factor concept” originally postulated for the CNS might also apply for the ENS: The neurotrophic factor concept in its basic form envisages that innervated tissues produce a signal (neurotrophic factor) specifically directed towards the innervating neurons which express receptors for the target-derived neurotrophic factor for the selective limitation of neuronal death occurring during development [27,28]. Translated into the present context, this would mean that the innervated tissue, i.e. the intestinal smooth muscle, produces GDNF which is acting on its corresponding receptors (Gfrα1 and RET) expressed by innervating nerves, i.e. myenteric neurons, to maintain their survival in adulthood – as recently confirmed by Rodrigues et al. for the rat intestine [29]. Consequently, a decreased amount of neurotrophic factor supply as evidenced by significantly reduced mRNA levels of GDNF would result in a loss of enteric neurons.

Effect of GDNF on Neuronal Survival and Differentiation of Postnatal Neurons

GDNF treatment of enteric nerve cell cultures enhanced both neuronal numbers and the formation of an extensive nervous network after one week of culture demonstrating that GDNF effectively promotes the survival and differentiation of postnatal myenteric neurons. Consistent with these results, Rodrigues et al. could demonstrate after 72 h of incubation enhanced neuronal survival, axonal outgrowth and nerve fiber fasciculation induced by GDNF using co-cultures of neonatal rat myenteric entires, smooth muscle and glial cells [29]. Similar effects have been reported in cell culture models of embryonic enteric neurons, in which a dose of 100 ng/ml GDNF promoted survival and proliferation of RET-expressing E14.5 neural crest cells [34]. According to the present data, other reports have demonstrated increased survival of myenteric neurons derived from rats of postnatal day 1 or 7 following GDNF treatment [35]. Since some enteric neuronal precursors do not withdraw from the cell cycle until postnatal day 14 [36], these cells might represent a pool of pluripotent cells in the postnatal gut responsible for the GDNF-induced increase in neuronal cell numbers. Alternatively, the survival promoting effect of GDNF might be due to a reduction of cell death as demonstrated for dopaminergic neurons of the substantia nigra [37] or motoneurons [38]. In addition, the observed GDNF induced formation of an extensive neuronal network in enteric nerve cell cultures is consistent with the induction of axonal growth in the peripheral nervous system mediated by GDNF [39]. These findings might delineate a GDNF-induced mechanism on regulating neuronal proliferation and differentiation in postnatal myenteric neurons that extends beyond the role of GDNF during development as demonstrated by knock-out animals [16,40,41]. The neurotrophic effect of GDNF on postnatal enteric neurons on the one hand and the decrease of GDNF in the muscularis propria of patients with DD on the other hand may provide a mechanism to explain the observed hypoganglionosis observed in this condition.

Effect of GDNF on Enteric Neuronal Plasticity

In addition to neuronal proliferation and differentiation GDNF also promoted the formation of synaptic vesicles in enteric nerve cell cultures as demonstrated by the upregulation of the synaptic vesicle marker synaptophysin. Outgrowing neuronal processes displayed multiple granular accumulations of synaptophysin resembling sites of synaptic communications also known as autonomic nerve fiber varicosities. These observations are consistent with a report of Zeng et al. [42] who described enhanced synaptic communication by modulating potassium currents and response to serotonin of cultured myenteric neurons following GDNF treatment. The increased expression of the synaptic vesicle marker synaptophysin induced by GDNF might...
Conclusion

Taken together, our results demonstrate for the first time that DD is associated with an increase in the neurotrophic factor GDNF. The in vitro data illustrate that GDNF promotes differentiation and synaptic plasticity of postnatal enteric neurons arguing for a role in the maintenance of the ENS during postnatal life. The compromised GDNF system in patients with DD may help to explain the shortfall of enteric neurons in this condition and further strengthens the hypothesis that the pathogenesis of DD may be linked – amongst other etiological factors – to an underlying enteric neuropathy.

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Supporting Information

Table S1 Primer sequences.

Author Contributions

Conceived and designed the experiments: M. Böttner KHS TW. Performed the experiments: M. Barrenschee IH JH J-HE TB DZ. Analyzed the data: M. Böttner KHS M. Barrenschee IH JH J-HE DB TW. Contributed reagents/materials/analysis tools: JH-E TB M. Böttner DZ. Wrote the paper: M. Böttner KHS TW. Carefully revised the manuscript and acknowledged the final version: M. Barrenschee IH J-HE TB DZ KHS TW M. Böttner.

References

1. Jun S, Stollman N (2002) Epidemiology of diverticular disease. Best Pract Res Clin Gastroenterol 16: 529–542.
2. Simpson J, Spiller R (2002) Colonic diverticular disease. Clin Evid: 436–444.
3. Böttner M, Wedel T (2012) Abnormalities of neuromuscular anatomy in diverticular disease. Dig Dis 30: 19–23.
4. Strate LL, Lii TL, Aldoori WH, Syngal S, Giovannucci EL (2009) Obesity increases the risk of diverticulitis and diverticular bleeding. Gastroenterology 136: 115–122 e111.
5. Humes DJ, Simpson J, Smith J, Sutton P, Zaitoun A, et al. (2012) Visceral hypersensitivity in symptomatic diverticular disease and the role of neuropetides and low grade inflammation. Neurogastroenterol Motil 24: 318–e163.
6. Simpson J, Sunfled F, Humes DJ, Jenkins D, Scholfield JH, et al. (2009) Post inflammatory damage to the enteric nervous system in diverticular disease and its relationship to symptoms. Neurogastroenterol Motil 21: 847–e838.
7. Dedovitch O, Salahzad E, Tanelis A, Pavlikis D, Pauwels N, et al. (2008) Morphologic pattern of myenteric neural plexus in colonic diverticular disease: A whole-mount study employing histochemical staining for acetylcholinesterase. Am J Anat 190: 525–530.
8. Goldie M, Burleigh DE, Belai A, Ghali L, Ashby D, et al. (2003) Smooth muscle cholinergic denervation hypersensitivity in diverticular disease. Lancet 361: 1945–1951.
9. Ivase H, Sadahiro S, Mukoyama S, Makouchi H, Yasuda M (2005) Morphology of myenteric plexuses in the human large intestine: comparison between large intestines with and without colonic divertica. J Clin Gastroenterol 39: 674–678.
10. Wedel T, Busing V, Heinrichs G, Nohroudi K, Bruch HP, et al. (2010) Diverticular disease is associated with an enteric neuropathy as revealed by morphometric analysis. Neurogastroenterol Motil 22: 407–414, e493–404.
11. Bassotti G, Battaglia E, Spinozzi F, Pelli MA, Tonini M (2001) Twenty-four hour recordings of colonic motility in patients with diverticular disease: evidence for abnormal motility and propulsive activity. Dis Colon Rectum 44: 1014–1820.
12. Knowles CH, De Giorgio R, Kapur RP, Bruder E, Farragia G, et al. (2010) The London Classification of gastrointestinal neuromuscular pathology: report on behalf of the Gastro 2009 International Working Group. Gut 59: 802–817.
13. Liu LF, Dobony DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260: 1130–1132.
14. Böttner M, Krieglstein K, Unsicker K (2000) The transforming growth factor-β system: a new tool to investigate site-specific gene expression in enteric ganglia of the human intestine. Neurogastroenterol Motil 22: 168–172, e152.
15. Schäfer KH, Saffrey MJ, Burnstock G, Mestres-Ventura P (1997) A new method for the isolation of myenteric plexus from the newborn rat gastrointestinal tract. Brain Res Brain Res Protoc 1: 109–113.
16. Schäfer KH, Mestres P, Marx P, Rose-John S (1999) The IL-6/sIL-6R fusion protein hyper-IL-6 promotes neurite outgrowth and neuron survival in cultured enteric neurons. J Interferon Cytokine Res 19: 527–532.
17. Painter MN, Burkit DP (1971) Diverticular disease of the colon: a deficiency disease of Western civilization. Br Med J 2: 450–454.
18. Peer A, Barrett PR, Park D, Rogers AJ, Galanko JA, et al. (2012) A high-fiber diet does not protect against asymptomatic diverticulosis. Gastroenterology 142: 266–272 e261.
19. Shen L, Pichel JG, Mayelli T, Sariola H, Lu B, et al. (2002) Gdnf haploinsufficiency causes Hirschsprung-like intestinal obstruction and early-onset lethality in mice. Am J Hum Genet 70: 435–447.
20. Du F, Wang L, Qian W, Liu S (2009) Loss of enteric neurons accompanied by decreased expression of GDNF and PI3k/Akt pathway in diabetic rats. Neurogastroenterol Motil 21: e1299–e1114.
21. von Boyen GB, Schuler N, Pfuger C, Spaniol U, Hartmann G, et al. (2011) Distribution of enteric glia and GDNF during gut inflammation. BMC Gastroenterol 11: 3.
22. Oppehennou RW (1991) Cell death during development of the nervous system. Ann Rev Neurosci 14: 451–501.
23. Korsching S (1993) The neurotrophic factor concept: a reexamination. J Neurosci 13: 2739–2748.
24. Rodriguez DM, Li YJ, Nair DG, Blemershasser MG (2011) Glial cell line-derived neurotrophic factor is a key neurotroph in the postnatal enteric nervous system. Neurogastroenterol Motil 23: e44–56.
25. Pachnis V, Mankoo B, Costantini F (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119: 1005–1017.
26. Anto-Bitach T, Abuel M, Gravid M, Delezide AL, Aug J, et al. (1998) Expression of the RET proto-oncogene in human embryos. Am J Med Genet 80: 481–486.
27. Golden JP, DeMarco JA, Osborne PA, Milbrandt J, Johnson EM Jr. (1999) Expression of neuritin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 158: 504–528.
28. Usaku T, Jain S, Yonemura S, Uchiyama Y, Milbrandt J, et al. (2007) Conditional ablation of GFRA1 in postmigratory enteric neurons triggers unconventional neuronal death in the colon and causes a Hirschsprung’s disease phenotype. Development 134: 2171–2181.
29. Heucker RH, Lampe PA, Johnson EM, Milbrandt J (1998) Neuritin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev Biol 200: 116–129.
30. Schäfer KH, Mestres P (1999) The GDNF-induced neurite outgrowth and neuronal survival in dissociated myenteric plexus cultures of the rat small intestine decreases postnatally. Exp Brain Res 125: 447–452.
31. Pham TD, Gershon MD, Rothman TP (1991) Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. J Comp Neurol 314: 789–788.
37. Burke RE (2006) GDNF as a candidate striatal target-derived neurotrophic factor for the development of substantia nigra dopamine neurons. J Neural Transm Suppl: 41–45.
38. Zhao Z, Alam S, Oppenheim RW, Previte DM, Evenson A, et al. (2004) Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Exp Neurol 190: 356–372.
39. Keller-Peck CR, Feng G, Sanes JR, Yan Q, Lichtman JW, et al. (2001) Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J Neurosci 21: 6136–6146.
40. Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, et al. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382: 73–76.
41. Sanchez MP, Silos-Santiago I, Friesen J, He B, Lara SA, et al. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382: 70–73.
42. Zeng F, Watson RP, Nash MS (2010) Glial cell-derived neurotrophic factor enhances synaptic communication and 5-hydroxytryptamine 3a receptor expression in enteric neurons. Gastroenterology 138: 1491–1501.
43. Burgoyne RD, Morgan A (2011) Chaperoning the SNAREs: a role in preventing neurodegeneration? Nat Cell Biol 13: 8–9.
44. Brose N, O’Connor V, Skehel P (2010) Synaptopathy: dysfunction of synaptic function? Biochem Soc Trans 38: 443–444.