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Enteric Neuronal Density Contributes to the Severity of Intestinal Inflammation

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Abstract

Background & Aims—Enteric neurons have been reported to be increased in inflamed regions of the bowel in patients with inflammatory bowel disease (IBD) or intestinal neuroangliomatosis. It is impossible to determine whether this hyperinnervation predates intestinal inflammation, results from it, or contributes to its severity in humans, so we studied this process in mice.

Methods—To determine whether the density of enteric neurons determines the severity of inflammation, we studied transgenic mice that have greater-than-normal (*Hand2*^{+/-} mice) or fewer-than-normal (NSE-noggin mice, which overexpress noggin under the control of the neuron-specific enolase promoter) numbers of neurons in the enteric nervous system (ENS). Colitis was induced with trinitrobenzene sulfonic acid or dextran sulfate sodium and the intensity of the resulting inflammation in *Hand2*^{+/-} and NSE-noggin mice was compared with that of wild-type littermates.

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Results—Severity of each form of colitis (based on survival, symptom, and histologic scores; intestinal expression of genes that encode proinflammatory molecules; and levels of neutrophil elastase and p50 NF- κ B *Hand2*+/- mice and significantly increased in NSE-noggin animals. Neither mouse differed from wild-type in the severity of delayed-type hypersensitivity (edema, T-cell and neutrophil infiltration, or expression of interleukin- α - -dinitro-1-fluorobenzene. Transgene effects on inflammation were therefore restricted to the gastrointestinal tract.

Conclusion—The severity of intestinal inflammation is associated with the density of the enteric innervation in mice. Abnormalities in ENS development might therefore contribute to the pathogenesis of IBD.

Keywords

IBD; intestinal inflammation; signaling; Crohn's disease; colitis; mice; DSS; TNBS

Introduction

The origin of inflammatory bowel disease (IBD) is unknown. IBD has been postulated to be associated with a genetically determined immune dysregulation and/or defect in gut structure that increases the probability that an environmental perturbation, such as altered bacterial composition, initiates inflammation¹. The enteric nervous system (ENS) regulates gastrointestinal (GI) motility and secretion; however, by affecting innate and adaptive immunity, the ENS also helps to protect the gut from infection^{2,3} and may thus participate in enteric IBD pathogenesis⁴⁻⁶.

An unexplained overabundance of enteric neurons has been observed in inflamed regions of the bowel in IBD^{4,5}. Conceivably, this hyperinnervation occurs because inflammation induces stem cells, which are retained in the mature intestine⁷, to generate new neurons⁸; however, neither pro- nor anti-inflammatory cytokines have been reported to induce enteric neurogenesis. An alternative, compatible with a genetic predisposition to IBD, is that an excess of enteric neurons pre-exists and either accentuates inflammation or predisposes the bowel to it. Such an association of enteric hyperinnervation with inflammation also occurs in intestinal ganglioneuromatosis^{9,10}, which resembles Crohn's disease.

We tested the hypothesis that enteric neuronal density is a determinant of the severity of intestinal inflammation. This hypothesis is difficult to evaluate in IBD because it is impossible to know how many neurons were present in the bowel prior to inflammation. In contrast, it is possible to ascertain the effect of the density of the enteric innervation on inflammation in animals because that can be set to be high or low before inflammation occurs. Genetic models were thus used to obtain mice in which the enteric innervation is more or less dense than normal. The bowel is hyperinnervated in transgenic mice that overexpress the bone morphogenetic protein (BMP) antagonist, noggin, directed to developing neurons by the NSE promoter (NSE-noggin mice)^{11,12}. BMPs promote enteric neuronal differentiation at the expense of continued precursor proliferation. Noggin-mediated BMP antagonism, therefore, extends the period of precursor proliferation in NSE-noggin mice and causes increases the ultimate number (~150%) of enteric neurons. Although NSE-noggin mice survive and gain weight normally, the proportions of some

subtypes of enteric neuron are abnormal and intestinal motility is irregular. A hypoplastic ENS is found in mice that are haploinsufficient for *Hand2*¹³, which encodes a basic helix–loop–helix transcription factor¹⁴ that is required for terminal differentiation of enteric neurons¹⁵. Transcripts encoding *Hand2* are significantly decreased in *Hand2*^{+/-} mice and the number of enteric neurons is reduced to about 59% of that of their wild-type (WT) littermates¹³. *Hand2*^{+/-} mice survive and gain weight normally; however, in the *Hand2*^{+/-} animals, colonic motility is decreased and gastrointestinal (GI) transit time is increased.

Rectal administration of 2, 4, 6 trinitrobenzene sulfonic acid (TNBS) or addition of dextran sulfate sodium to drinking water (DSS) were used to induce colitis in NSE-noggin and *Hand2*^{+/-} mice, as well as in their WT littermates. The severity of the colitis was quantified as a function of time following instillation of TNBS or administration of DSS. For both types of inflammation, intensity was found to be significantly greater in NSE-noggin mice, and less in *Hand2*^{+/-} animals, than that of WT littermates. These observations support the hypothesis that the pre-existent enteric neuronal density affects the severity of intestinal inflammation, with hyperplasia being pro- and hypoplasia anti-inflammatory. These studies are consistent with the idea that the ENS contributes to the pathogenesis of IBD⁴⁻⁶.

Materials and Methods

Animals

Breeding pairs of *NSE-noggin* mice (CD-1 background) were obtained from Dr. John A. Kessler at the Feinberg School of Medicine, Northwestern University. *Hand2*^{+/-} mice (C57Bl/6 background) were obtained from Dr. Peter Cserjesi at Tulane University. Animals were bred at Columbia University. Because the backgrounds of NSE-noggin (CD-1) and *Hand2*^{+/-} mice (C57/Bl6) differed, each of these animals were their own WT littermate controls.

Experimental colitis

Colitis was induced with TNBS (100 mg/kg)¹⁶ or DSS (5% in drinking water)^{17, 18} as previously described. During treatment, a clinical disease activity index was computed daily¹⁹. This index was based on changes in body weight, graded on a 5-point scale, stool consistency, graded on a 3-point scale, and blood in stools, graded on a 3-point scale. Colons were removed, following euthanasia with CO₂ asphyxiation, seven days after infusion of TNBS or saline and 6 days after starting DSS. Transverse sections (5 µM) of paraffin-embedded distal colon (3 cm) were stained with hematoxylin and eosin. An expert pathologist, blinded to each animal's treatment, analyzed the tissue. Inflammatory cell infiltration and ulceration were rated on 3-point scales while crypt damage was scored on a 5-point scale to give a maximum histological score of 11¹⁹.

RT-PCR

RNA was extracted with Trizol (Invitrogen) and treated with DNase I (1U/ml). PCR, utilizing primers for β-actin, confirmed absence of DNA contamination. Reverse transcriptase (High Capacity cDNA Archive Kit; Applied Biosystems) was used to convert 1 µg of sample to cDNA. RT-PCR was employed to quantify messenger RNA encoding IFNγ,

TNF α and CCR5. Expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were purchased from Applied Biosystems (Foster City, CA). The real-time reaction contained cDNA (5 μ l), primers for the cytokine/chemokine/standard (250 nmol), PCR master mix (12.5 μ l; Applied Biosystems, Foster City, CA) and nuclease-free water (6.25 μ l). A GeneAmp 7500 sequence detection system (Applied Biosystems) was used to quantify cDNA levels. Duplicates were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 cycles of annealing at 60°C for 20 sec, extension at 60°C for 1 min, and denaturation at 95°C for 15 sec. TaqMan 7500 software was used for data analysis.

PCR microarray

Focused PCR microarrays (Inflammatory Cytokines; Superarray; SABiosciences; Frederick, MD) were used. cDNA samples were mixed with sufficient master mix to be loaded into the wells of 96-well PCR-array plates. Microarrays were carried out and plates were read using a TaqMan 7500 PCR machine. A web-based integrated PCR Array Expression Analysis Suite provided by SaBiosciences was employed for image analysis and data acquisition. Intensity of signals was normalized to GAPDH.

Immunoblots and ELISA

Colonic segments were homogenized in cell lysis buffer (Boston Bioproducts), boiled in Laemmle buffer²⁰, and 30 mg of the resulting protein lysate was subjected to electrophoresis in a 10% Tris-Glycine gel. Following transfer to an Immobilon-P membrane (Millipore), blots were incubated for 12 hrs at 4° C with polyclonal antibodies to neutrophil elastase or p50 NF κ B (diluted 1:1000; Santa Cruz Biotechnology; Santa Cruz, CA). Bound primary antibodies were located with secondary antibodies labeled with horseradish peroxidase (diluted 1:2000, incubation for 1 hr; Santa Cruz). Chemiluminescence was used to detect peroxidase activity (Supersignal West Pico Chemiluminescent Substrate, Pierce). Blots were stripped (Restore buffer; Pierce) and re-probed with goat antibodies to GAPDH (Santa Cruz) for standardization. Results were quantified using ImageQuant software (Amersham). R&D Systems kits were used for ELISA.

Immunocytochemistry

Ears, thyroid, pancreas, spleen, kidney, and distal colon from 6-8 week old *Hand2*^{+/-}, *NSE-noggin* mice and their WT littermates were fixed for 3 hrs with 4% formaldehyde (from paraformaldehyde) in 0.2 M phosphate buffer at pH 7.4. Tissues were cryoprotected (30% sucrose; 4° C), embedded in Neg50™ (Richard Allan Scientist, Kalamazoo, MI), frozen with liquid N₂, and sectioned in a cryostat-microtome. Sections were collected on positively charged slides (Superfrost™; Fisher Scientific). Primary and secondary antibodies were applied sequentially²¹; DNA was stained with bisbenzimidazole (1 μ g/ml) and slides were mounted in 50% glycerol in 0.5 M bicarbonate buffer (pH 8.6).

Delayed type hypersensitivity (DTH)

DTH was induced with 2,4-dinitro-1-fluorobenzene (DNFB). Three *Hand2*^{+/-} and 3 *NSE-noggin* mice (age 6-8 wks), as well as 3 of each of their respective WT littermates were

sensitized to DNFB (20 μ l of a 0.2% solution in acetone and olive oil), topically applied to shaved abdominal skin, on each of two days. On day 5, mice were challenged by rubbing 0.2% DNFB (10 μ l) onto both sides of the left ear. The base without DNFB was applied to both sides of the right ear. The thickness of each ear was measured with a micrometer 24 hours after challenge in order to assess swelling. Mice were euthanized and real-time PCR was used to quantify the abundance of transcripts encoding IL1 β , Ifn γ , and TNF α in half of each ear. The remaining half ear was fixed and used for immunocytochemical demonstration of dendritic cells, macrophages and T-cells.

Statistical analyses

Student's t test and one-way ANOVA were used, respectively, to compare single and multiple means. Two-way ANOVA was used to analyze the significance of the contributions to observed variation of time (days) and genotype (*Hand2*^{+/-} and *NSE-noggin* vs respective controls) to the clinical course of TNBS- and DSS-induced colitis.

Results

Severity of TNBS-induced colitis in *Hand2* haploinsufficient and WT mice

Clinical scores, composed of the change in weight, stool blood, and stool consistency were determined daily for seven days after induction of TNBS-induced colitis in *Hand2*^{+/-} mice and WT littermates. The combined total scores (Fig. 1A) were used to compare the two types of mouse. Two-way ANOVA was used to test the significance of days (time) and genome (*Hand2*^{+/-} vs *WT*) as sources of variation. Both were extremely significant and suggest that *WT* and *Hand2*^{+/-} mice become increasingly sick as a function of time after instillation of TNBS; however, the clinical condition of *WT* mice deteriorates more than that of *Hand2*^{+/-} littermates. Survival was also significantly greater in *Hand2*^{+/-} than in *WT* mice (Fig. 1B). Significant differences between *WT* and *Hand2*^{+/-} mice were observed, not only total scores (Fig. 1A), but also the components, loss of weight (Fig. 1C), stool blood (Fig. 1D) and stool consistency (Fig. 1E) increased significantly more as a function of time after TNBS colitis in *WT* than in *Hand2*^{+/-} animals.

The effect of the *Hand2*^{+/-} genotype on intestinal inflammation was not restricted to TNBS-induced colitis. DSS-induced colitis was also more severe in *WT* than in *Hand2*^{+/-} littermates, not only in total scores but also in each component, weight loss, stool blood, and stool consistency (Supplemental Table 1; two-way ANOVA). Scores increased significantly over the 6-day period in both *WT* and *Hand2*^{+/-} mice ($p < 0.0001$ for all); moreover, genotype (*WT* > *Hand2*^{+/-}) was a significant contributor to each score's variation.

The expression of genes encoding molecules involved in the mediation or regulation of colonic inflammation was investigated to validate the clinical suggestion that TNBS-induced colitis is less severe in *Hand2*^{+/-} mice than in *WT* littermates. Focused microarrays were used to enable expression of a large number of genes in inflammatory pathways to be analyzed simultaneously. In the absence of TNBS-induced colitis, there was little difference between *WT* and *Hand2*^{+/-} mice in colonic expression of genes encoding pro- or anti-inflammatory molecules (Fig. 2A); however, after induction of TNBS-colitis, expression of

94% of genes encoding pro-inflammatory molecules in WT mice exceeded that in *Hand2*^{+/-} littermates (Fig. 2B). Differences in expression of 61% of these genes reached significance ($p < 0.05$). Real-time PCR was used to confirm these data. Transcripts encoding TNF α , CCR5, and IFN γ (Fig. 2C) were each significantly more abundant during TNBS-induced colitis in WT mice than in *Hand2*^{+/-} littermates. ELISA-determined levels of the proinflammatory cytokines, IL-1 β , IL-6 and TNF α were also significantly more elevated in WT than *Hand2*^{+/-} mice (Fig. 2D). Similar elevations of cytokines (WT > *Hand2*^{+/-}) were observed in DSS colitis (Supplemental Fig. 1). These observations support the idea that Hand2 haploinsufficiency confers resistance to colitis.

To verify that differences in expression of cytokines were reflected in differences in expression of protein markers of inflammation, immunoblots were quantified densitometrically to evaluate neutrophil elastase and p50 NF κ B (Fig. 3). Levels in vehicle (30% ethanol in PBS)-treated animals were taken to represent constitutive expression, which was compared to that after TNBS-induced colitis. Constituent levels of neutrophil elastase and p50 NF κ B did not differ significantly between WT and *Hand2*^{+/-} mice. In both WT and *Hand2*^{+/-} animals, moreover, TNBS-induced colitis significantly increased neutrophil elastase (Fig. 3B) and p50 NF κ B (Fig. 3C); however, neutrophil elastase and p50 NF κ B were each significantly more elevated following instillation of TNBS in WT mice than in *Hand2*^{+/-} animals. These observations verify that Hand2 haploinsufficiency in mice confers resistance to TNBS-induced colitis.

Severity of TNBS-induced colitis in NSE-noggin and WT mice

Total clinical scores and those of its component parameters in homozygous *NSE-noggin* mice were compared to those of WT littermates daily following the initiation of TNBS-induced colitis. As with *Hand2*^{+/-} mice, two-way ANOVA was employed to test the hypotheses that total scores and individual parameters increased over the 7-day period following instillation of TNBS and that the genomic difference between WT and NSE-noggin littermates contributed significantly to the observed variation. The severity of TNBS-induced colitis was significantly greater in *NSE-noggin* than in WT mice, not only in total scores (Fig. 4A), but also in mortality (Fig. 4B), and in those of the component parameters, weight loss, stool blood, and stool consistency (Fig. 4C). After 7 days, histological signs of inflammation in the colons of WT mice were not as severe (Fig. 4D) as in NSE-noggin littermates (Fig. 4E). The total score, degree of ulceration, and crypt damage were each significantly greater in *NSE-noggin* than in WT mice (Fig. 4F). These observations suggest that NSE-noggin mice are significantly more sensitive to TNBS-induced colitis than are WT animals. A similar study, carried out with mice given DSS for 6 days, also revealed that both the clinical course and the histological effects of DSS-induced colitis were significantly more severe in *NSE-noggin* than in WT mice (Supplemental Table 1).

The expression of genes encoding molecules involved in the mediation or regulation of inflammation was investigated to test the clinical suggestion that the severity of TNBS-induced colitis is greater in *NSE-noggin* mice than in WT littermates. Focused microarrays with >70 pro-inflammatory pathway-related genes were used. Gene expression in NSE-noggin mice was compared with that in their WT littermates under constitutive (baseline)

conditions (Fig. 5A) and after TNBS-induced colitis (Fig. 5B). No differences between *WT* and *NSE-noggin* mice could be detected in the constitutive expression of genes encoding pro- or anti-inflammatory molecules (Fig. 5A); however, after the induction of TNBS-colitis, expression of 75% of the genes (55) encoding pro-inflammatory molecules in *NSE-noggin* mice exceeded that in *WT* littermates (Fig. 5B). Differences were significant ($p < 0.05$) in 43% of the genes. Levels of three proinflammatory cytokines, IL1 β (Fig 5C), IL6 (Fig 5D) and TNF α were measured to verify that differences in gene expression were reflected in proteins. All were increased significantly more in *NSE-noggin* than in *WT* mice. Cytokine protein concentration increased similarly (*NSE-noggin* > *WT*) during DSS-induced colitis (Supplementary Fig. 2).

Immunoblotting was used to verify that differences between *WT* and *NSE-noggin* mice in expression of cytokines were reflected in differences in expression of protein markers of inflammation (Fig. 6A). No difference between *WT* and *NSE-noggin* mice was observed in constituent levels of either neutrophil elastase (Fig. 6B) or p50 NF κ B (Fig. 6C) immunoreactivities in the colon. TNBS significantly increased levels of both proteins (Fig. 6 B, C); however, the TNBS-induced colitis-associated increment in both neutrophil elastase (Fig. 6B) and p50 NF κ B (Fig. 6C) was significantly greater in *NSE-noggin* than in *WT* mice.

Severity of DNFB-induced contact hypersensitivity in *Hand2* haploinsufficient and *NSE-noggin* mice

The studies outlined above indicate that the severity of TNBS-induced colitis is reduced in *Hand2*^{+/-} mice and increased in *NSE-noggin* mice. Before the differences in susceptibility of *Hand2*^{+/-} and *NSE-noggin* mice to TNBS-induced colitis can be attributed to the difference between them in numbers of enteric neurons, however, it is necessary to know whether the altered sensitivity of the bowel to inflammation in each strain is gut-specific. No evidence of autoimmunity was detected in either *Hand2*^{+/-} or *NSE-noggin* mice; the histological appearance of the kidneys, thyroid gland, pancreas, and spleen of neither *Hand2*^{+/-} nor *NSE-noggin* mice differed from that in their respective *WT* littermates and numbers of T cells, macrophages, and dendritic cells were comparable in the thyroid and pancreas (data not illustrated). DNFB-induced delayed-type hypersensitivity (DTH) was thus studied to test the null hypothesis that a systemic alteration in susceptibility to inflammation occurs in *Hand2*^{+/-} and/or *NSE-noggin* mice. Immediately preceding the application of DNFB or vehicle, and again after 24 hrs, the thickness of DNFB- and vehicle-treated ears was measured with a micrometer to estimate edema. Because vehicle caused a slight, but significant, increase in ear thickness, the DNFB-induced increment in ear thickness was estimated from the ratio of the thickness of the DNFB-treated ear to that of its paired vehicle-treated control (Fig. 7A, B). Significant increases in ear thickness occurred in the DNFB-treated ears in all *WT* mice as well as in their *NSE-noggin* (Fig. 7A), and *Hand2*^{+/-} littermates (Fig. 7B); however, the magnitude of the DNFB-induced increment in ear thickness in neither *NSE-noggin* (Fig. 7A) nor *Hand2*^{+/-} mice (Fig. 7B) differed significantly from that in respective *WT* littermates.

The abundance of transcripts encoding the proinflammatory cytokines, IL1 β , IFN γ , and TNF α , in the DNFB-treated and control ears were evaluated to confirm the data obtained

from measurements of ear thickness (Supplemental Fig. 3). Abundance of transcripts in the DNFB-treated ear was again normalized to that in paired vehicle-treated controls. Although transcripts encoding each cytokine increased significantly in the DNFB-treated ears of every mouse, the magnitudes of the DNFB-induced increases in *NSE-noggin* and *Hand2*^{+/-} mice did not differ significantly from those in respective *WT* littermates. Finally, the effects of DNFB on the numbers per mm² of CD3-immunoreactive T cells and neutrophil elastase-immunoreactive neutrophils were analyzed in immunocytochemically stained sections through each ear (Fig. 7C). The infiltration of CD3- and neutrophil elastase-immunoreactive cells was significantly greater in the DNFB-treated than in the control ear of every mouse (Fig. 7C); nevertheless, no significant differences were observed between *WT* mice and their *NSE-noggin* or *Hand2*^{+/-} littermates.

Discussion

We tested the hypothesis that ENS hyperplasia contributes to the severity of intestinal inflammation. This hypothesis was originally suggested by clinical reports of increased numbers of enteric neurons in inflamed regions of the bowel in IBD^{4,5} and studies that have associated neuronal genes, such as *LRRK2*²² and *Ninjurin2*²³, with an increased risk of IBD. The hypothesis predicts that intestinal inflammation would be more severe in mice with a hyperplastic ENS and less severe in animals with a hypoplastic ENS. *Hand2*^{+/-} and *NSE-noggin* mice were selected for study, not only because the ENS is hyperplastic in *NSE-noggin* and hypoplastic in *Hand2*^{+/-} animals, but also because there was reason to believe that their genetic abnormalities would affect the ENS but not their immune systems.

Hand2 is expressed in extraembryonic connective tissue, the heart, and autonomic neurons²⁴. *Hand2* expression appears to be necessary for terminal differentiation of enteric¹⁵ and sympathetic neurons^{25,26} and for development of right ventricular cardiac muscle²⁷. Although *Hand2* deletion is lethal in mice at about E10.5, *Hand2*^{+/-} animals survive, have a normal lifespan, and are fertile¹³. Effectors of innate or acquired immunity, moreover, do not express *Hand2*. *NSE-noggin* mice overexpress the BMP antagonist, noggin^{11,12} but noggin overexpression is delayed until neurons or endocrine cells, which express the NSE promoter, develop. The transgene thus does not interfere with early effects of BMPs on neural crest or gut and its expression is relatively confined to sites of NSE expression, which include the ENS. Despite ENS hyperplasia and irregular GI motility, *NSE-noggin* mice gain weight normally and are fertile.

TNBS and DSS were employed to induce colitis. Although TNBS-induced colitis resembles Crohn's disease and DSS-induced colitis resembles ulcerative colitis, neither is a faithful mimic of IBD; nevertheless, TNBS, is a haptene that combines with endogenous proteins or luminal antigens to evoke a transmural TH1-mediated colitis²⁸. DSS- differs from TNBS-induced colitis in being more mucosal and dependent on innate immunity¹⁷. The colitis induced, either with TNBS or DSS, was more severe in *NSE-noggin* mice and less severe in *Hand2*^{+/-} animals than in their respective *WT* littermates. The reciprocal alteration of the severity of inflammation in *NSE-noggin* and *Hand2*^{+/-} animals was evident in clinical scores, survival, histological scores, abundance of transcripts encoding cytokines, cytokine protein levels, neutrophil infiltration, and p50 NFκB expression. Focused microarrays,

moreover, revealed that the TNBS-induced increase in the expression of multiple proinflammatory genes was exacerbated in *NSE-noggin* animals and attenuated in *Hand2*^{+/-} mice. These data are consistent with the tested hypothesis that the severity of inflammation varies directly with the pre-existing numbers of enteric neurons. The opposite effects of ENS hyperplasia and hypoplasia on both TNBS- and DSS-induced colitis and the disparate natures of the genetic abnormalities that give rise to them, furthermore, mutually support the idea that it is the number of enteric neurons that modulates the severity of inflammation. This conclusion, however, requires that the genetic perturbations used to modulate numbers of enteric neurons not exert confounding effects on systemic immunity or manifestations of inflammation.

DNFB was used to induce DTH in an ear to determine whether confounding effects on immunity or inflammation are present at an extra-enteric site in *NSE-noggin* or *Hand2*^{+/-} mice. DTH severity was reflected in the manifestation of edema (ear thickness), expression of cytokines (IL1 β , IFN γ , and TNF α), and infiltration of T cells and neutrophils. Although DNFB reproducibly evoked DTH, none of the measures of severity in either *NSE-noggin* or *Hand2*^{+/-} mice differed significantly from those in *WT* littermates. These observations support the ideas that the transgenes affect the severity of inflammation only in the gut and that these effects are secondary to the respective ENS hyperplasia or hypoplasia that they cause.

It is not yet clear how enteric neuronal density affects the severity of intestinal inflammation. Effectors of both innate and acquired immunity express receptors for neurotransmitters/neuromodulators. Mononuclear leukocytes, dendritic cells, and macrophages, for example, express muscarinic acetylcholine receptors M₁-M₅, as well as nicotinic receptor subunits α ₂, α ₅, α ₆, and α ₇ ²⁹. Nicotinic stimulation (of α ₇ subunits) is anti-inflammatory ³⁰ and probably accounts for intestinal anti-inflammatory properties of vagus nerve stimulation ³¹. Lymphocytes and macrophages also express numerous serotonin receptor subtypes ³²; moreover, serotonin can initiate DTH ³³, stimulate CD4 cells to secrete IL-16 ³⁴, and promote lymphocyte proliferation ³⁵. Potentiation of serotonin in serotonin transporter knockout mice increases the severity of colitis induced both by TNBS ³⁶ and by IL-10 deletion ³⁷. In contrast, knockout of tryptophan hydroxylase-1, which depletes mucosal serotonin, decreases the severity of colitis ¹⁸. Neuroimmunomodulation, moreover, appears to affect interactions between the intestinal mucosa and pathogenic bacteria ³; moreover, neuropeptides exert effects that are either pro- or anti-inflammatory ⁶. Neurons thus are probably able to influence the severity of inflammation through release of neurotransmitters/neuromodulators. Because inflammation is more severe when the ENS is hyperplastic and less severe when the ENS is hypoplastic, the net effect of the ENS is probably pro-inflammatory. Observations are thus consistent with the possibility that a pre-existing regional ENS hyperplasia predisposes to inflammation. Alternatively, ENS hyperplasia may increase the severity of inflammation even when it is provoked by an ENS-independent perturbation, such as the intestinal flora. Further studies are needed to determine whether any of the genes that are known to regulate ENS development are altered in patients with IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Morbidity and mortality of TNBS-induced colitis are reduced in *Hand2*^{+/-} mice
 A. Time-action curve showing total clinical scores as a function of time after TNBS. Two-way ANOVA was used to test the significance of days and genome (*Hand2*^{+/-} vs *WT*) as sources of variation. B. Mortality (n = 6 mice/group). C-E. Time action curves for loss of weight (C), stool blood (D), and stool consistency (E). Two-way ANOVA was again used to test the significance of days and genome as sources of variation. Severity of colitis was significantly greater in *WT* than in *Hand2*^{+/-} animals for all measured parameters.

Fig. 2. Transcripts encoding molecules related to inflammation are less abundant in the colon during TNBS-induced colitis in *Hand2*^{+/-} mice than in WT littermates

A. Baseline expression in a focused microarray. The abundance of transcripts in WT mice is plotted logarithmically on the ordinate as a function of the abundance of the same transcripts in *Hand2*^{+/-} mice plotted logarithmically on the abscissa. The central line shows the best fit of the data and the parallel lines above and below depict 95% confidence limits. One chemokine (C-C motif) ligand 8 (circle above the upper 95% confidence limit) is expressed to a significantly greater extent in WT than in *Hand2*^{+/-} littermates. All other genes examined at baseline are similarly expressed in WT and *Hand2*^{+/-} colon. B. TNBS-induced colitis; data plotted as in A. Many genes (filled circles below the lower 95% confidence limit) are expressed to a significantly greater extent in WT than in *Hand2*^{+/-} mice. C. After TNBS induction of colitis, the abundance of transcripts encoding TNFα, chemokine (C-C motif) receptor 5, and IFNγ is greater in WT than in *Hand2*^{+/-} mice. D. ELISA-determined levels of IL1β, IL6, and TNFα are all significantly higher in WT than in *Hand2*^{+/-} mice.

Fig. 3. Levels of markers of inflammation are lower in *Hand2*^{+/-} than in *WT* mice
 P50 NFκB and neutrophil elastase in mouse colon quantified by immunoblotting under constitutive conditions and after TNBS-induced colitis. A. Representative immunoblots. GAPDH = loading control. B. Neutrophil elastase to GAPDH ratio in the colon under control conditions and after TNBS-induced colitis. Increase in neutrophil elastase during TNBS-induced colitis in *Hand2*^{+/-} << *WT* mice. C. p50 NFκB to GAPDH ratio in the colon under constitutive conditions and after TNBS-induced colitis. Increase in p50 NFκB during TNBS-induced colitis *Hand2*^{+/-} << *WT* mice.

Fig. 4. Morbidity and mortality of TNBS-induced colitis are increased in *NSE-noggin* mice
Two-way ANOVA was used to test the significance of days and genome (*NSE-noggin* vs WT) as sources of variation ($n = 6$ mice/group) in clinical measures of colitis. A. Time-action curve for total clinical scores. B. Mortality. C. Time-action curves for components of total scores, including weight loss, stool blood, and stool consistency. Severity of colitis was significantly greater in *NSE-noggin* than in WT animals for total scores, loss of weight, stool blood, and stool consistency. D-F. Histological evidence of TNBS-induced colitis in *NSE-noggin* mice $\gg$ WT littermates; manifested in total scores, crypt damage and ulcerations. D. WT colon; 7 days following TNBS-induced colitis. Minimal signs of inflammation are evident. E. *NSE-noggin* colon; 7 days following TNBS-induced colitis. The mucosa is ulcerated (arrows) and a submucosal infiltrate of inflammatory cells (E; arrow) can be discerned. F. Quantitation of the data. The markers = 100 μ m (D and E left) and 20 μ m (E right). mm = muscularis mucosa.

Fig. 5. Molecules related to inflammation are more abundantly expressed in the colon during TNBS-induced colitis in *NSE-noggin* than in *WT* mice

A. Constitutive expression in a focused microarray. The abundance of transcripts in *WT* mice is plotted logarithmically on the ordinate as a function of the abundance of the same transcripts in *NSE-noggin* mice plotted logarithmically on the abscissa. The central line shows the best fit of the data and the parallel lines depict 95% confidence limits. No significant differences can be seen in the expression of any of the genes included in the array. B. TNBS-induced colitis. The data are plotted as in A. Thirty genes (filled circles above the upper 95% confidence limit) are significantly more expressed in *NSE-noggin* mice than in *WT* littermates. C-E. Levels of IL-1 β (C), IL-6 (D), and TNF α (E) are all increased significantly more in *NSE-noggin* than in *WT* mice.

Fig. 6. Levels of inflammatory markers are higher in *NSE-noggin* than in *WT* mice

A. Representative immunoblots. GAPDH = loading control. B. Neutrophil elastase to GAPDH ratios in the colon in *NSE-noggin* and *WT* mice were not significantly different under constitutive conditions, but the increase after TNBS-induced colitis in *NSE-noggin* >> *WT* mice. C. Constitutive levels of p50 NFκB in *NSE-noggin* and *WT* mice were not significantly different. The increase in p50 NFκB after TNBS-induced colitis in *NSE-noggin* >> *WT* mice.

Fig. 7. The severity of extra-enteric inflammation differs from WT in neither *Hand2*^{+/-} nor *NSE-noggin* mice

DTH was induced in one ear with DNFB. The contralateral vehicle-treated ear served as a control. A. DNFB-induced change in ear thickness compared in WT and *NSE-noggin* mice. The pretreatment ratio of the DNFB-treated to control ear was not significantly different from 1; *NSE-noggin* $\approx$ WT. One day post-treatment, swelling in the DNFB-treated $\gg$ control ear again, *NSE-noggin* $\approx$ WT. B. DNFB-induced change in ear thickness compared in WT and in *Hand2*^{+/-} mice. The pretreatment ratio of the DNFB-treated to control ear was not significantly different from 1; *Hand2*^{+/-} $\approx$ WT. One day post-treatment, swelling in the DNFB-treated $\gg$ control ear; again, *Hand2*^{+/-} $\approx$ WT. C, D. T cells and neutrophil infiltration one day post-DNFB-induced DTH. Immunocytochemically demonstrated CD3⁺ T cells (Fig. 7C) and neutrophil elastase⁺ neutrophils (Fig. 7D; insets above graphs). The densities (per mm²) of T cells and neutrophils in the DNFB-treated $\gg$ control ears in all animals; however, the densities of neither in *NSE-noggin* nor *Hand2*^{+/-} mice differed significantly from those in WT littermates. Markers = 100 μ m (CD3) and 20 μ m (neutrophil elastase).