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Expression Level of *Hand2* Affects Specification of Enteric Neurons and Gastrointestinal Function in Mice

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Abstract

Background & Aims—*Hand2* is a basic helix-loop-helix transcription factor required for terminal differentiation of enteric neurons. We studied *Hand2* haploinsufficient mice, to determine whether reduced expression of *Hand2* allows sufficient enteric neurogenesis for survival but not for development of a normal enteric nervous system (ENS).

Methods—Enteric transcripts that encode *Hand2* and the neuron-specific embryonic lethal abnormal vision proteins HuB, HuC, and HuD were quantified. Immunocytochemistry was used to identify and quantify neurons. Apoptosis was analyzed with the TUNEL procedure. Intracellular

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microelectrodes were used to record inhibitory junction potentials. Gastrointestinal transit and colonic motility were measured in vivo.

Results—Levels of enteric *Hand2* transcripts were associated with genotypes of mice, in the following order: *Hand2*^{+/+} > *Hand2LoxP*^{+/+} > *Hand2*^{+/-} > *Hand2LoxP*^{-/-}. Parallel reductions were found in expression of HuD and, in regional and phenotypic manners, numbers of neurons; numbers of nNOS⁺ and calretinin⁺, but not substance P⁺ or vasoactive intestinal peptide⁺ neurons, decreased. No effects were observed in stomach or cecum. Apoptosis was not detected, consistent with the concept that *Hand2* inhibits neuronal differentiation, rather than regulates survival. The amplitude of inhibitory junction potentials in colonic circular muscle was similar in *Hand2* wild-type and haploinsufficient mice, although in haploinsufficient mice, the purinergic component was reduced and a nitroergic component appeared. The abnormal ENS of haploinsufficient mice slowed gastrointestinal motility but protected mice against colitis.

Conclusion—Reduced expression of factors required for development of the ENS can cause defects in the ENS that are subtle enough to escape detection yet cause significant abnormalities in bowel function.

Keywords

transcriptional regulation; nervous system development; ELAV; mouse model

INTRODUCTION

Hand1 and *Hand2*, which encode basic helix-loop-helix transcription factors¹, are expressed in developing gut^{2,3}. Enteric expression of *Hand2* in mice is developmentally regulated and restricted to crest-derived cells, while that of *Hand1* occurs in muscle and interstitial cells of Cajal. Although deletion of *Hand2* does not interfere with the colonization of the bowel by crest-derived cells, these cells are unable to form neurons in vitro³. Transfection of enteric crest-derived cells (ENCDC) with siRNA to silence *Hand2* in vitro also prevents neuronal differentiation. The Wnt1-Cre-mediated conditional inactivation of *Hand2* in migrating crest-derived cells interferes with the terminal differentiation of HuD-expressing enteric neurons; nevertheless, enteric crest-derived precursors express early pan-neuronal markers, such as β 3-tubulin. It has thus been suggested that *Hand2* expression is required for terminal differentiation of enteric neurons, albeit not for ENCDC to colonize the bowel, commit to a neuronal lineage, or form glia.

Exon 1 of *Hand2* was flanked with loxP sites to generate conditional knockout mice³. Although *Hand2*^{LoxP/LoxP} mice appear normal, a cleft palate develops in *Hand2*^{LoxP/-} fetuses, suggesting that flanking intron 1 of the *Hand2* gene with loxP sites generates a hypomorphic allele that impairs development, at least of the palate. Similarly, in vitro experiments with siRNA have suggested that there is a minimum essential threshold of *Hand2* expression that must be exceeded for terminal differentiation of enteric neurons³.

We now show that enteric neurogenesis is a quantitative function of *Hand2* expression, which is reduced in the bowel of *Hand2*^{+/-} and further reduced in *Hand2*^{LoxP/-} mice. Parallel reductions occur in the enteric expression of the neuron-specific gene, *HuD*, and in the numbers of enteric neurons, especially those containing neuronal nitric oxide synthase (nNOS) and calretinin. The gene-dosage of *Hand2* thus appears to be critical for the acquisition of a normal complement of enteric neurons. These molecular defects have functional consequences. Gastrointestinal (GI) motility is reduced in *Hand2* haploinsufficient mice, which are also, paradoxically, resistant to intestinal inflammation.

MATERIALS AND METHODS

Animals and measures of motility

Hand2^{+/-} mice were bred at Columbia University. The “floxed” *Hand2* allele includes LoxP sites placed 5' of the start of transcription and within the first intron⁴. Mutant embryos were identified by PCR genotyping of extraembryonic membranes. A non-absorbed dye was used to measure total transit time, the time required to eject a glass bead placed into the rectum was employed to evaluate colonic motility, and conventional intracellular microelectrodes were employed to analyze inhibitory junction potentials in smooth muscle (see Supplemental Methods for details).

Real-time PCR

RNA extraction and cDNA preparation were as previously described⁵. cDNA was amplified using sequence-specific primers (see supplemental data, Table 1). PCR products were sequenced and found to match the appropriate sequences in the GenBank. Real-time PCR was used to quantify transcripts extracted from mouse gut (SYBR Green I; LightCycler, Roche Molecular Biochemicals, Indianapolis, IN).

Immunocytochemistry and histochemistry

Tissues were fixed with 4% formaldehyde (from paraformaldehyde) in 0.2 M phosphate buffer at pH 7.4. Dissected bowel was fixed overnight at 4° C. Fixed preparations from E17 gut were then cryoprotected (30% sucrose; 4° C), embedded in Neg50™ (Richard Allan Scientist, Kalamazoo, MI), frozen (liquid N₂), and sectioned with a cryostat-microtome. Fixed lamina preparations containing the submucosal or longitudinal muscle with attached myenteric plexus (LMMP) were prepared from 6-8 week old mice by dissection and examined as whole mounts. Procedures used for immunostaining have been described previously⁶. Acetylcholinesterase activity was demonstrated histochemically⁷. (see Supplemental Methods for details.)

TUNEL assay

Apoptosis was detected in tissue fixed as above, according to the manufacturer's instructions, with the *TMR red In Situ Cell Death Detection Kit* (Roche, NJ), (supplemented with 0.1 % sodium citrate). Terminal transferase was omitted as a negative control. Tissue was exposed to DNase I prior to the assay (10 min; Roche, NJ) to provide a positive control.

TNBS-induced colitis

Acute colitis was induced with 2,4,6 trinitrobenzenesulfonic acid (TNBS; Fluka; 5mg/35 gram mouse)⁸ in 6 age-, sex- and weight-matched *Hand2*^{+/-} mice and 6 WT littermates. Mice were euthanized after seven days and tissue was taken for histological examination. The distal 2 cm of colon was embedded in paraffin and 5 μM transverse sections were stained with hematoxylin and eosin. An expert pathologist scored the histopathology without knowledge of the animals' treatment. Scoring was based on inflammation, crypt damage and ulceration⁹. (see Supplemental Methods)

Statistical analyses

One-way ANOVA or, when only 2 groups were studied, Student's t test was used to compare means. Equality of variances was analyzed with an F test and Welch's correction was employed when variances of populations was significantly different.

RESULTS

***Hand2* regulation of ENS development is gene dosage dependent**

We tested the hypothesis that, like the palate, the ENS is more sensitive than other targets to reduced levels of *Hand2* during development. We therefore analyzed the *Hand2*^{+/-} and *Hand2*^{LoxP/-} gut to determine whether hypomorphic effects could be detected in the ENS. The abundance of transcripts encoding *Hand2* in the E13 gut, analyzed by real-time PCR, reflected the animals' genotype and decreased in the rank order *Hand2*^{+/+} > *Hand2*^{LoxP/+} > *Hand2*^{+/-} > *Hand2*^{LoxP/-}. Differences between groups were significant. To determine whether *Hand2* expression dose-dependently affects neuronal development, transcripts encoding the neuron-specific proteins, HuB, HuC, and HuD were quantified in E13 gut (Fig. 1B-D). The abundance of transcripts encoding HuB, which was relatively low, did not differ significantly in *Hand2*^{+/+}, *Hand2*^{LoxP/+}, *Hand2*^{+/-}, and *Hand2*^{LoxP/-} animals (Fig. 1B). Although the enteric abundance of transcripts encoding HuC in *Hand2*^{LoxP/+} and *Hand2*^{+/-} mice was not different from that in WT animals, it was significantly lower in the bowel of *Hand2*^{LoxP/-} mice (Fig. 1C). In contrast, the abundance of enteric transcripts encoding HuD paralleled the abundance of transcripts encoding *Hand2*; transcripts encoding HuD decreased in the order *Hand2*^{+/+} > *Hand2*^{LoxP/+} > *Hand2*^{+/-} > *Hand2*^{LoxP/-} (Fig. 1D). Differences between groups were significant.

The effects of *Hand2* haploinsufficiency on the generation and/or survival of neurons in the mature ENS was evaluated by counting the number and determining the size of neurons in the bowel of 6-8 week old *Hand2*^{+/+} and *Hand2*^{+/-} mice. Antibodies to HuD were used to identify neurons immunocytochemically (Fig. 2A, B). Because of the cleft palates that develop in *Hand2*^{LoxP/-} mice, these animals do not survive and thus their postnatal bowel could not be investigated. The numbers of neurons, per ganglion (Fig. 2C) or per mm² (Fig. 2D) were significantly lower in *Hand2*^{+/-} mice at 6-8 wks than in their *Hand2*^{+/+} littermates. Similar changes in neuronal numbers were observed at 3 wks, although ganglia were less well organized; the numbers in *Hand2*^{+/-} mice were 73.5 ± 6.4 % that of *Hand2*^{+/+}; p < 0.001). Neurons were smaller in *Hand2*^{+/-} than in *Hand2*^{+/+} mice at 6 but not 3-wks of age. The relative abundance of neurites and connectives, evaluated in LMMP preparations in which β3-tubulin immunoreactivity (Fig. 2E, F) or AChE (Supplemental Fig. 1) activity was demonstrated, were also found to be lower (and space between connectives greater) in the myenteric plexus of *Hand2*^{+/-} than WT mice. As with neurons visualized with antibodies to HuD, fewer neurons could be detected with antibodies to β3-tubulin in myenteric ganglia of *Hand2*^{+/-} than in *Hand2*^{+/+} mice (Supplemental Fig. 2A, B, E). Neurons were smaller and their cytoplasm not as well-filled with β3-tubulin immunoreactivity in the *Hand2*^{+/-} neurons as in their *Hand2*^{+/+} counterparts (compare Supplemental Figs. 2A and B). In contrast to neurons, glia were not decreased in myenteric ganglia of *Hand2*^{+/-} animals (Supplemental Fig. 2 C, D, E, and F). The glia to neuron ratio was thus greater in *Hand2*^{+/-} than in *Hand2*^{+/+} mice (Supplemental Fig. 2G). These data suggest that both the number and eventual sizes of neurons in the postnatal ENS parallels the abundance of transcripts encoding HuD, which in turn depends upon the abundance of transcripts encoding *Hand2*.

Enteric nNOS-expressing neurons are reduced in *Hand2* haploinsufficient mice

We investigated the possibility that differentiation of subsets of enteric neurons might be more sensitive than others to the reduction of *Hand2* expression that occurs in the *Hand2*^{+/-} or *Hand2*^{LoxP/-} mice and fail to develop in the ENS of these animals. The nature of the defect in the ENS associated with *Hand2* haploinsufficiency, therefore, might be reflected in a change in the "chemical coding" of the ENS¹⁰. Enteric neurons of different phenotypes are born at different ages¹¹; moreover, enteric neuronal phenotypes reflect the number of

proliferative division precursors undergo prior to exiting the cell cycle¹². Particularly because terminal differentiation is *Hand2*-dependent, we used *Hand2* haploinsufficient mice to test the hypothesis that the development of early- and late-born types of enteric neuron, which have, respectively, undergone fewer or greater numbers of proliferative divisions, differentially depend on *Hand2* regulation.

Cholinergic neurons, which coexpress calretinin, are born early while many neurons that contain nNOS are born late¹². Vasoactive intestinal peptide (VIP), which is coexpressed with nNOS¹⁰, is found in late-born neurons¹¹, although nitrergic neurons can also be detected as early as E11.5¹³⁻¹⁵. The effects of *Hand2* haploinsufficiency on the development of calretinin- and nNOS-immunoreactive (⁺) neurons were investigated in the gut of *Hand2*^{+/+}, *Hand2*^{LoxP/+}, *Hand2*^{+/-}, and *Hand2*^{LoxP/-} mice at E17. Hu immunoreactivity was used as a neuronal marker. The densities of nNOS⁺ neurons, normalized to that of Hu⁺ cells, were significantly lower in the intestines of *Hand2*^{+/-} and *Hand2*^{LoxP/-} mice than in those of *Hand2*^{+/+} or *Hand2*^{LoxP/+} animals (Fig. 3A). The reduction of the density of nNOS⁺ neurons in the *Hand2*^{LoxP/-} and *Hand2*^{+/-} gut, furthermore, was due to a decrease in the numbers of nNOS⁺ neurons, rather than to a reduction in the intensity of nNOS immunoreactivity in individual cells (Fig. 3B-E). The numbers (as % *Hand2*^{LoxP/+}) of Phox2b⁺ ENCDC (106 ± 7 ; n = 6), BrdU-labeled cells 2 hrs after administration of BrdU to pregnant dams (104 ± 9 ; n = 6), and the ratio of BrdU-labeled to Phox2b⁺ cells (98 ± 4 ; n = 6) in the foregut at E12 of *Hand2*^{LoxP/-} mice did not differ significantly (p = 0.67) from those in *Hand2*^{LoxP/+} animals. It thus seems unlikely that *Hand2* haploinsufficiency impairs the colonization of the bowel by ENCDC or their subsequent proliferation. Surprisingly, the decrease in relative density of nNOS⁺ neurons in *Hand2*^{LoxP/-} gut relative to *Hand2*^{LoxP/+} controls was regionally specific in that it was seen in the small intestine, proximal, and distal colon, but not in the stomach or cecum (Fig. 3F, G). Some loss of cecal nNOS⁺ neurons might be demonstrable if more animals were to be examined. In contrast to nNOS⁺ neurons, the density of substance P (the best known tachykinin)-immunoreactive neurons (examined as an example of a late appearing neuronal phenotype^{16,17}) in *Hand2*^{LoxP/-} mice was not significantly different from that of *Hand2*^{LoxP/+} mice (Fig. 3H; n = 9 mice). Because of the smaller number of nNOS⁺ neurons, the nNOS⁺/substance P⁺ ratio was significantly greater in *Hand2*^{LoxP/-} than in *Hand2*^{LoxP/+} mice (Fig. 3I). The effect of *Hand2* haploinsufficiency on the development of nNOS⁺ and calretinin⁺ neurons, studied in the gut of *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} mice at E18, was found to be remarkably similar. For both types of neuron, there was a preservation of development in the stomach (Fig. 4A) and cecum (Fig. 4B); however, development of nNOS and calretinin-containing neurons was markedly deficient in the small intestine (Fig. 4C) and the colon (Fig. 4D). Effects of the reduction of *Hand2* expression are thus selective. These observations are consistent with the idea that phenotypic expression in the ENS is *Hand2*-dependent and that the level of *Hand2* expression necessary to support the acquisition of a full neurotransmitter-defined neuronal phenotype varies as a function of neuronal subtype and bowel region.

The reduction of nNOS and calretinin-expressing neurons in *Hand2* haploinsufficient mice was not confined to fetal gut, but persisted through postnatal weeks 6-8. Significantly fewer nNOS and calretinin-expressing neurons were found in the ENS of *Hand2*^{+/-} than in *Hand2*^{+/+} intestine at 6 weeks of age (Supplemental Fig. 2); moreover, the deficit in each type of neuron was of equal magnitude in both the submucosal (nNOS = 50.4 ± 4.6 % WT; p < 0.001; calretinin = 31.9 ± 2.2 % WT; p < 0.001) and myenteric plexus (nNOS = 55.6 ± 4.6 % WT; p < 0.001; calretinin = 32.9 ± 2.9 % WT; p < 0.001) (Supplemental Fig. 2). *In situ* hybridization and immunocytochemistry were used to investigate transcripts encoding VIP to determine whether the nNOS deficiency in *Hand2*^{+/-} gut is due to an absence of a type of neuron or a failure of an existing neuron to acquire its full terminally differentiated

phenotype (Supplemental Fig. 4). VIP transcripts, but not immunoreactivity, were detected at E14 (not illustrated)¹⁶; however, both were found at E18 in *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} mice. At that age, nNOS and VIP immunoreactivities were mostly coincident in *Hand2*^{LoxP/+} mice but not in the *Hand2*^{LoxP/-} ENS. Because VIP immunoreactivity in neuronal perikarya is inconsistent in the absence of colchicine treatment, in situ hybridization was used to quantify VIP-expressing and nNOS⁺ neurons in the proximal small intestines of the same *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} animals (Supplemental Fig. 4C; n = 3). Despite the significantly smaller number of nNOS⁺ neurons in the *Hand2*^{LoxP/-} gut, the number of VIP-expressing neurons did not differ in *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} mice. As a result, there was a significant increase in the ratio of VIP-expressing to nNOS⁺ neurons in the *Hand2*^{LoxP/-} bowel (Supplemental Fig. 4D).

Apoptosis is not detectable in enteric neurons of newborn or postnatal mice

Because the myenteric plexus of mature *Hand2*^{+/-} mice contains fewer neurons than that of their *Hand2*^{+/+} littermates, it is possible that decreased *Hand2* expression adversely affects enteric neuronal survival. The density of HuD-expressing neurons in *Hand2*^{+/+} and ^{+/-} mice was found to be the same at E17, but to decline in *Hand2*^{+/-} mice at 3 and 6 weeks of age; therefore, apoptosis was compared in the myenteric plexuses of 3 week-old *Hand2*^{+/+} and *Hand2*^{+/-} mice. The TUNEL assay was used for this purpose in LMMP preparations (Fig. 5). The immunoreactivity of HuD was used as a neuronal marker and DNA was stained with bisbenzimidazole. The TUNEL assay failed to detect apoptosis in myenteric neurons either in *Hand2*^{+/+} mice (Fig. 5A-D, K) or in their *Hand2*^{+/-} littermates (Fig. 5E-H, L). In contrast, in a positive control treated with DNAase before the TUNEL assay, all of the nuclei in both smooth muscle and ganglia showed the red fluorescence indicative of DNA fragmentation (Fig. 5I). No non-specific TUNEL staining was detected when terminal transferase was omitted (Fig. 5J). These observations suggest that if apoptosis had been present in myenteric neurons, the TUNEL assay would have revealed it. Similar results were obtained with the TUNEL assay in frozen sections cut through the gut of newborn *Hand2*^{+/+} or *Hand2*^{+/-} mice (data not illustrated). Again, no evidence of apoptosis was detected in myenteric ganglia of either type of animal; therefore, *Hand2* haploinsufficiency does not seem to lead to apoptotic death of neurons through the first 3 weeks of life.

Enteric motility is abnormal in *Hand2*^{+/-} mice

Because *Hand2*^{+/-} mice are fertile and survive with a typical lifespan, they have been considered to be “normal”. There are, nevertheless, significant reductions in the enteric expression of *Hand2* (Fig. 1A) and HuD (Fig. 1D), while numbers of total, nNOS⁺ and calretinin⁺ enteric neurons are also decreased (Fig. 3A-G, 4). ENS development, therefore, is abnormal in *Hand2*^{+/-} mice even though the severity of the defects does not lead to pseudoobstruction or to the death of the animals. To determine whether these defects have functional consequences, we measured total GI transit time, colonic motility, and IJPs in *Hand2*^{+/-} mice. *Hand2*^{LoxP/-} mice die at birth; therefore, these animals cannot be used to study postnatal intestinal motility.

Total gastrointestinal transit time¹⁸ was significantly greater in *Hand2*^{+/-} than in *Hand2*^{+/+} mice (Fig. 6A). Because gastric emptying is included in total gastrointestinal transit time, and nNOS⁺ neurons were not affected in the stomachs of *Hand2*^{+/-} mice, colonic motility was separately evaluated. Colonic motility was significantly slower in *Hand2*^{+/-} than in *Hand2*^{+/+} mice (Fig. 6B). These data confirm that *Hand2* haploinsufficiency not only alters numbers and phenotypes of enteric neurons, but also causes gastrointestinal motility to become abnormal.

We investigated IJPs to determine whether the defect in colonic motility in *Hand2*^{+/-} mice could be related to a defect in nitrenergic inhibition of smooth muscle. IJPs are thought to be mediated in part by NO¹⁹, although prior studies in mouse colon have suggested that inhibitory neurotransmission is purinergic, rather than nitrenergic^{20,21}. IJPs were recorded in the circular muscle of the colons of *Hand2*^{+/+} (Fig. 6C) and *Hand2*^{+/-} mice (Fig. 6D). The NOS inhibitor, N ω -nitro-L-arginine (LNNA) did not affect IJPs in *Hand2*^{+/+} mice (Fig. 6C). The IJP, however, was almost completely eliminated by apamin, which blocks the small conductance Ca²⁺-activated K⁺ channels upon which purinergic hyperpolarization of smooth muscle depends. In contrast, LNNA significantly reduced the amplitude of IJPs in *Hand2*^{+/-} mice and IJPs were eliminated in the presence of LNNA plus apamin (Fig. 6D). In the absence of inhibitors, IJP amplitude was approximately the same in *Hand2*^{+/+} and *Hand2*^{+/-} mice. These findings suggest that IJPs are predominantly purinergic in *Hand2*^{+/+} mice, but in *Hand2*^{+/-} animals, IJPs have a nitrenergic as well as a purinergic component.

***Hand2* haploinsufficient mice are protected from inflammation**

Motility of the bowel is thought to protect the intestine from infection and inflammation²². Because motility is abnormal in *Hand2*^{+/-} mice, we studied the susceptibility of the colon of *Hand2*^{+/-} mice to tissue damage from experimental inflammation. TNBS was used induce colitis. Clinical symptoms, including weight loss, diarrhea, and blood in the stool, were followed. Animals were permitted to survive for 7 days after the administration of TNBS after which the colons were fixed to permit the severity of the inflammation-induced tissue damage to be quantified histologically. Scores were assigned, on a scale of zero to three, taking account of ulceration, crypt damage and leukocyte infiltration²³ (Fig. 7A, B). Despite the slower intestinal transit and colonic motility, the extent of tissue damage following the induction of colitis with TNBS was significantly less in *Hand2*^{+/-} mice than in their *Hand2*^{+/+} littermates (Fig. 7A-C).

DISCUSSION

Prior studies with *Hand2*^{+/-} mice have assumed that the bowel of these animals is not abnormal because the mice survive and breed. On the contrary, we now find that substantial ENS abnormalities occur in *Hand2*^{+/-} mice, which while not lethal, affect bowel function. *Hand2* expression is reduced by about half in *Hand2*^{+/-} animals and more so in *Hand2*^{LoxP/-} mice; these reductions are associated with a decrease in HuD expression. Hu proteins affect neuronal differentiation²⁴⁻²⁶. HuD is an RNA binding protein that is related to the ELAV (Embryonic Lethal Abnormal Vision) proteins of *Drosophila*, which are required for nervous system development and maintenance²⁷. ELAV proteins extend the longevity of mRNAs by binding to AU-rich elements in the 3' untranslated region, thereby enhancing their stability²⁸. Within neurons, HuD competes with other RNA binding proteins that primarily destabilize transcripts²⁷. Because of this balance, reductions of HuD expression, secondary to decreased expression of *Hand2* in *Hand2*^{+/-} mice, might have the effect of destabilizing transcripts, which would, when sufficiently severe, interfere with neuronal differentiation and/or survival. In adult *Hand2*^{+/-} animals, these changes in gene expression result in an ENS that has fewer and smaller neurons than in wild-type mice; the defects in the ENS could be due to a direct effect of *Hand2* on neuronal differentiation/survival or they may be indirect and secondary to the associated decrease in expression of HuD.

Prior in vitro experiments using siRNA to disrupt *Hand2* function suggested that there is a threshold for *Hand2* expression, below which neurogenesis fails³. Current observations support this suggestion, however, they also indicate that such a threshold cannot be the same for all classes of enteric neuron. *Hand2* haploinsufficiency interferes with the development of nNOS⁺ and calretinin⁺ neurons but not with that of substance P⁺ neurons; moreover,

subsets of nNOS⁺ and calretinin⁺ neurons in the stomach and cecum evidently can develop and survive even when *Hand2* is 30-50% of normal (in hypomorphic and haploinsufficient animals), while most of those in the remainder of the intestine cannot. It appears, therefore, that the nNOS⁺ and calretinin⁺ neuronal sets are heterogeneous with regard to the levels of *Hand2*, and probably also HuD, expression required to support their development/survival.

Apoptosis was not detected in the postnatal bowel of either *Hand2*^{+/+} or *Hand2*^{+/-} mice. The TUNEL assay, which was employed for this investigation, was able to detect DNA fragmentation when it was induced as a positive control prior to the procedure; moreover, the use of LMMP preparations enable large regions and many neurons of the myenteric plexus to be examined. It is likely, therefore, that the failure of the TUNEL assay to detect apoptosis in 3 week-old animals of either genotype is due to the relative absence of apoptosis during normal ENS development²⁹, which is not altered by *Hand2* haploinsufficiency. The reduction of neurons that occurs in the *Hand2*^{+/-} ENS, in the absence of evidence for increased apoptosis, is compatible with the idea that the haploinsufficiency of *Hand2* interferes with terminal differentiation, rather than survival, of neurons. That likelihood is supported by the observation in *Hand2*^{LoxP/-}, but not *Hand2*^{LoxP/+} mice, that VIP expression occurs in neurons that lack nNOS (with a consequent increase in the VIP-expressing/nNOS⁺ ratio in hypomorphic animals). The nNOS component of the phenotype of neurons that normally contain both VIP and nNOS is not acquired but the incompletely developed VIP-expressing neurons do not die. Prior observations have also suggested that the deletion or silencing of *Hand2* interferes with the terminal differentiation of enteric neurons³. It remains formally possible, however, that non-apoptotic death of neurons³⁰ or early apoptotic death of a subset of precursors contributes to the deficient numbers of neurons found in the *Hand2*^{+/-} ENS.

The reduction of the total number of enteric neurons and alteration of the distribution of neurons of different phenotypes that occurs in adult *Hand2* haploinsufficient mice, moreover, was associated with abnormal gastrointestinal motility; total gastrointestinal transit time increased and colonic motility slowed. These motor malfunctions suggest that the changes in phenotypic expression within the ENS of *Hand2*^{+/-} mice disturb the intrinsic reflex circuits upon which motility depends. The observed deficiencies in numbers of nNOS⁺ and calretinin⁺ neurons in the small intestine and colon might contribute to the slowing of motility in *Hand2*^{+/-} mice; however, the maintenance of IJP amplitude and the surprising acquisition of a nitrergic component to IJPs in these animals suggest that the defect is not a simple loss of nitrergic inhibition of smooth muscle. It is thus likely that additional defects exist in the *Hand2*^{+/-} ENS, perhaps involving a purinergic deficiency and/or nitrergic and Ach/calretinin-expressing interneurons, which also contribute to the abnormal motility of the *Hand2*^{+/-} bowel.

The motility of the gut is thought to be important in protecting it from microbial invasion²². One might anticipate, therefore, that the decrease in motility associated with *Hand2* haploinsufficiency would predispose the bowel to inflammation, perhaps due to increased invasion by commensal organisms, which is thought to be a factor in the etiology of human inflammatory bowel disease^{31,32}. Instead, the *Hand2*^{+/-} phenotype was found to confer a resistance to the inflammation induced in the colon by instillation of TNBS. TNBS acts as a haptene, which binds to tissue and evokes a cellular immune response. Neuropeptides and neurotransmitters have been found to affect immune responses and inflammation³³. The change in neuronal numbers and the altered distribution of chemically-defined types of neuron that occur in *Hand2*^{+/-} mice could therefore confer resistance to inflammation by affecting the balance of pro- and anti-inflammatory neuropeptides and/or neurotransmitters that enteric neurons release. A net decrease in the drive to inflammation provided by the ENS may contribute to the resistance of *Hand2*^{+/-} mice to TNBS-induced colitis.

The observations that reductions in *Hand2* expression, which are relatively modest in magnitude, disturb the development of the ENS and the behavior of the mature bowel is of considerable interest. The defect in the *Hand2*^{+/-} gut is not an aganglionosis and the animals are clearly not a model for Hirschsprung's disease. In fact, a similar defect arising in the human gut would almost certainly not be noticed by a pathologist examining the bowel. Such diagnoses tend to be binary; ganglia are rated as present or absent³⁴. Only an investigator looking for it, as some have^{35,36}, would recognize a subtle decrease in numbers of neurons or in subsets expressing particular chemically-defined phenotypes, such as those defined by nNOS or calretinin immunoreactivities. In contrast, the pathophysiology of abnormally slow transit in most patients probably would not be diagnosed and, in fact, is common in chronic constipation or constipation-predominant irritable bowel syndrome. Constipation in the absence of an aganglionosis or myopathy is usually considered a functional gastrointestinal disease (FGID)³⁴. It is thus possible that FGID or subsets of it arise from subtle defects in the ENS resulting from genetic factors that reduce expression of genes such as *Hand2* or *HuD* that are required in ENS development. Similarly, subtle inherited alterations in the neuronal composition of the ENS might contribute individual variations in sensitivity to perturbations of the bowel that give rise to intestinal inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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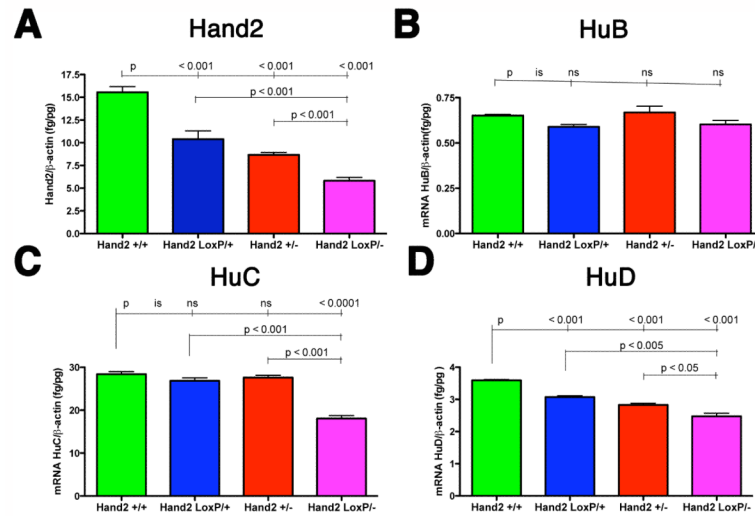


Figure 1. *Hand2*^{+/-} and *Hand2*^{LoxP/-} mice are hypomorphic
 (A-D) Real-time PCR was used to quantify transcripts encoding *Hand2*, HuB, HuC, and HuD in the gut of *Hand2*^{+/+}, *Hand2*^{LoxP/+}, *Hand2*^{+/-}, and *Hand2*^{LoxP/-} mice at E13. (A) The abundance of transcripts encoding *Hand2* in the gut of *Hand2*^{+/+} mice > *Hand2*^{LoxP/+} mice > *Hand2*^{+/-} mice > *Hand2*^{LoxP/-} mice. (B). No significant differences are found in abundance of enteric transcripts encoding HuB in *Hand2*^{+/+}, *Hand2*^{LoxP/+}, *Hand2*^{+/-}, and *Hand2*^{LoxP/-} mice. (C) The abundance of enteric transcripts encoding HuC is significantly decreased in *Hand2*^{LoxP/-} mice. (D) The abundance of enteric transcripts encoding HuD in *Hand2*^{+/+} > *Hand2*^{LoxP/+} > *Hand2*^{+/-} > *Hand2*^{LoxP/-}. Each parameter (A-D) was analyzed in 3 mice of each genotype

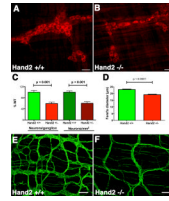


Figure 2. Neuronal numbers are reduced in the ENS of *Hand2*^{+/-} mice

HuD immunoreactivity in the myenteric plexus of the colon of (A) a *Hand2*^{+/+} mouse and (B) a *Hand2*^{+/-} littermate. C, D, E. Numbers of HuD-immunoreactive neurons were quantified and compared in *Hand2*^{+/+} and *Hand2*^{+/-} bowel as a function of ganglia (C) and area (D). Significantly more neurons are found in the myenteric plexus of the *Hand2*^{+/+} than in the *Hand2*^{+/-} gut, both counted as neurons per ganglia and neurons/mm². E. Neurons are significantly smaller in *Hand2*^{+/-} than in the *Hand2*^{+/+} colon.

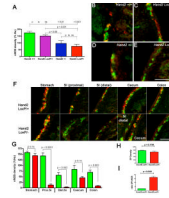


Figure 3. nNOS⁺ neurons are *Hand2*-dependent

(A-G) The density (pixels/mm gut length, normalized to the density of Hu⁺ neurons) of nNOS⁺ neurons was analyzed in the intestine of *Hand2*^{+/+}, *Hand2*^{LoxP/+}, *Hand2*^{+/-}, and *Hand2*^{LoxP/-} mice at E17. (A) Although the density of nNOS⁺ neurons in *Hand2*^{LoxP/+} was similar to that of *Hand2*^{+/+} controls, nNOS⁺ neuronal density was significantly reduced in *Hand2*^{+/-}, and *Hand2*^{LoxP/-} fetuses. (B-E) The myenteric plexus, doubly immunostained to demonstrate nNOS (red) and Hu immunoreactivities (green). B. *Hand2*^{+/+}. C. *Hand2*^{LoxP/+}. D. *Hand2*^{+/-}. E. *Hand2*^{LoxP/-}. The fluorescence of nitroergic neurons is yellow because antibodies to Hu and nNOS doubly immunostain these cells. The proportion of nNOS⁺ neurons to Hu⁺ neurons is about the same in *Hand2*^{+/+} and *Hand2*^{LoxP/+} gut but is reduced in *Hand2*^{+/-} and especially in *Hand2*^{LoxP/-} bowel. (F, G) The density of nNOS immunoreactivity in *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} mice, normalized to that of Hu, was compared in the stomach, proximal small intestine (SI prox), distal small intestine (SI dist), cecum, and colon. F. The myenteric plexus, doubly immunostained to demonstrate nNOS (red) and Hu (green) is illustrated for each of the regions analyzed in G. The regional specificity of effect of genotype on nNOS⁺ neuronal density is particularly evident in the panel illustrating the distal small intestine and cecum of the *Hand2*^{LoxP/-} mouse where a loop from each of these regions from the same animal appear in a single section; nNOS⁺ neurons are virtually absent in the distal small intestine but abundant in the cecum. G. Quantitation of the ratios of the regional density of nNOS to Hu immunoreactivities in the bowel of *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} mice. The nNOS⁺ neuronal density in the stomach and cecum was similar in *Hand2*^{LoxP/+} and *Hand2*^{LoxP/-} fetuses; however, in SI prox, SI dist, and colon the nNOS⁺ neuronal density in *Hand2*^{LoxP/+} was significantly greater than that in *Hand2*^{LoxP/-}. (H) The density of SP immunoreactivity/mm length of bowel in *Hand2*^{LoxP/+} proximal small intestine does not differ significantly from that in the gut of E17 *Hand2*^{LoxP/-} mice. I. The ratios of the densities of SP to nNOS immunoreactivity were measured in proximal small intestine. Because of the decrease in nNOS without a corresponding decrease in SP, the SP/nNOS ratio is significantly greater in *Hand2*^{LoxP/-} than *Hand2*^{LoxP/+} animals. Each parameter (A-G) was analyzed in 3 mice of each genotype. Five additional *Hand2*^{LoxP/+} and 4 *Hand2*^{LoxP/-} animals were analyzed in H-I. Scale bars: 50mm.

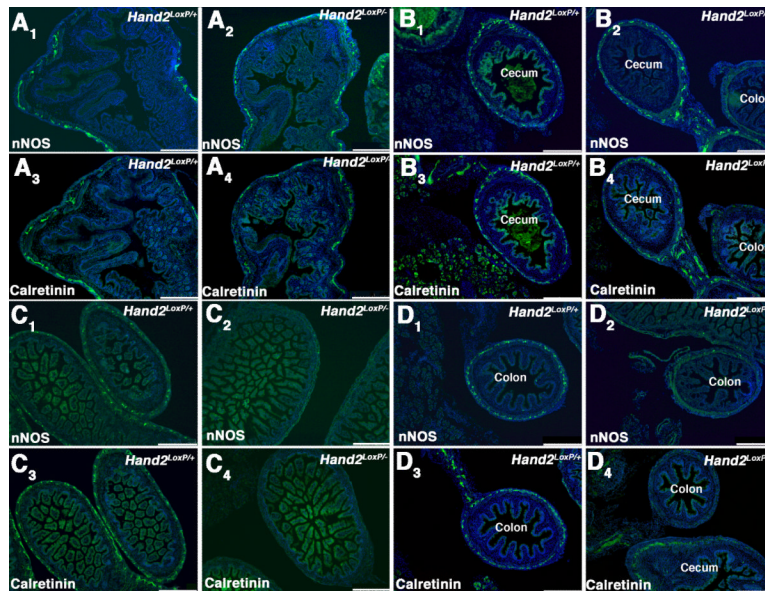


Figure 4. Regional effects of *Hand2* haploinsufficiency on enteric calretinin⁺ neurons and nNOS⁺ are similar

Representative sections are shown at low magnification to facilitate comparisons between nNOS⁺ and calretinin⁺ neurons at various levels of the E18 bowel of *Hand2*^{LoxP/+} (A₁, A₃, B₁, B₃, C₁, C₃, D₁, D₃) and *Hand2*^{LoxP/-} (A₂, A₄, B₂, B₄, C₂, C₄, D₂, D₄) mice. Two mice of each genotype are illustrated. A. Stomach. Numbers of nNOS⁺ and calretinin⁺ neurons are similar in *Hand2*^{LoxP/+} (A₁, A₃) and *Hand2*^{LoxP/-} (A₂, A₄) mice. B. Cecum. Numbers of nNOS⁺ and calretinin⁺ neurons are similar in *Hand2*^{LoxP/+} (B₁, B₃) and *Hand2*^{LoxP/-} (B₂, B₄) mice. The section in C₄ contains a segment of colon, which can be compared to the segment of cecum in the same field of view. There is little calretinin immunoreactivity in the colon, but calretinin immunoreactivity is prominent in the cecum of the same *Hand2*^{LoxP/-} animal. C. Small intestine. The immunoreactivities of nNOS and calretinin are prominent in the developing myenteric plexus in *Hand2*^{LoxP/+} (C₁, C₃) mice but deficient in that of *Hand2*^{LoxP/-} (C₂, C₄) littermates. D. Colon. nNOS and calretinin immunoreactivities are prominent in the developing myenteric plexus of *Hand2*^{LoxP/+} (D₁, D₃) mice but lacking in that of *Hand2*^{LoxP/-} (D₂, D₄) mice. D₄ contains cecum, which can be compared to colon in the same field. Calretinin immunoreactivity is evident in the cecum, but lacking in the colon of the same *Hand2*^{LoxP/-} animal. The bars = 50 μm.

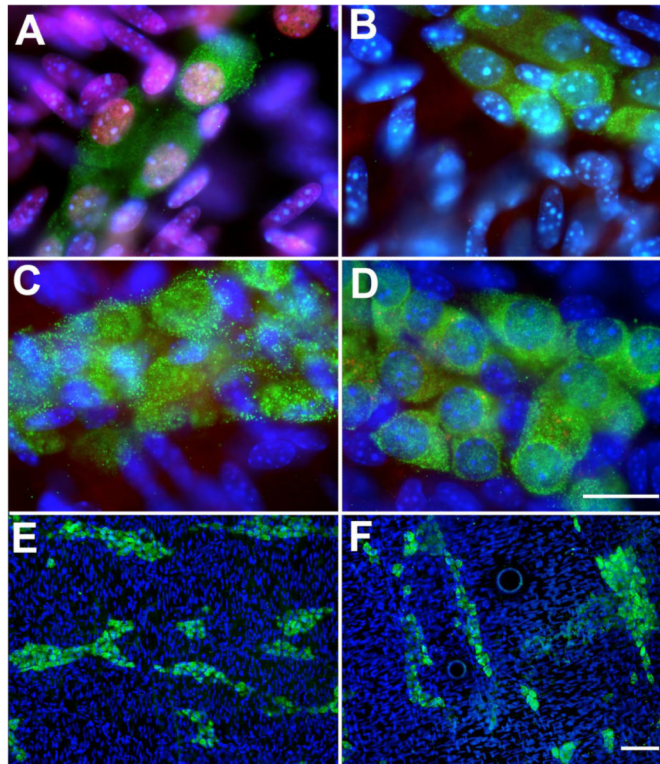


Figure 5. Little or no apoptosis of myenteric neurons is seen in either *Hand2*^{+/+} or *Hand2*^{+/-} mice

Laminar whole mounts of longitudinal muscle with adherent myenteric plexus were prepared from the bowel of 3 week-old *Hand2*^{+/+} and *Hand2*^{+/-} mice. The TUNEL procedure (red) was carried out to detect apoptosis in neurons identified as Hu⁺ cells (green). DNA was stained with bisbenzimidazole (blue). A-D. A field from a *Hand2*^{+/+} mouse, imaged to show the TUNEL reaction (A), DNA (B), Hu immunoreactivity (C) and merged images (D). No structures show TUNEL staining (A); therefore, no red fluorescence appears in the merged image (D). The blue fluorescence of smooth muscle nuclei (B) forms a bed to which the myenteric ganglia adhere (D). E-F. A field from a *Hand2*^{+/-} mouse, imaged to show the TUNEL reaction (E), DNA (F), Hu immunoreactivity (G) and the merged images (H). Again, no structures show TUNEL staining (E) and no red fluorescence appears in the merged image (H). More neurons are evident in the ganglia of *Hand2*^{+/+} mice (C, D) than in those of *Hand2*^{+/-} animals (G, H). I. The preparation was treated with DNAase to provide a positive control for the TUNEL reaction. A high power merged image is shown in which the DNA fragmentation is made evident by the superimposed merged red fluorescence of the TUNEL reaction and the blue fluorescence of DNA. Hu⁺ neurons are marked by their green fluorescence. J. Terminal transferase was omitted to provide a negative control for the TUNEL reaction. A merged image is shown at the same magnification as that of the positive control (I). There is no red fluorescence; therefore, nuclei display unmodified blue fluorescence in the merged image. Again, Hu⁺ neurons are marked by their green fluorescence. K. A high power merged image of a myenteric ganglion from a *Hand2*^{+/+} mouse subjected to the TUNEL procedure. No red fluorescence indicative of DNA fragmentation has been produced by the TUNEL assay. L. A high power merged image of myenteric ganglion from a *Hand2*^{+/-} mouse subjected to the TUNEL procedure. Again, no red fluorescence indicative of apoptosis has been produced by the TUNEL assay. The markers A-H = 100 μ m; I-L = 20 μ m.

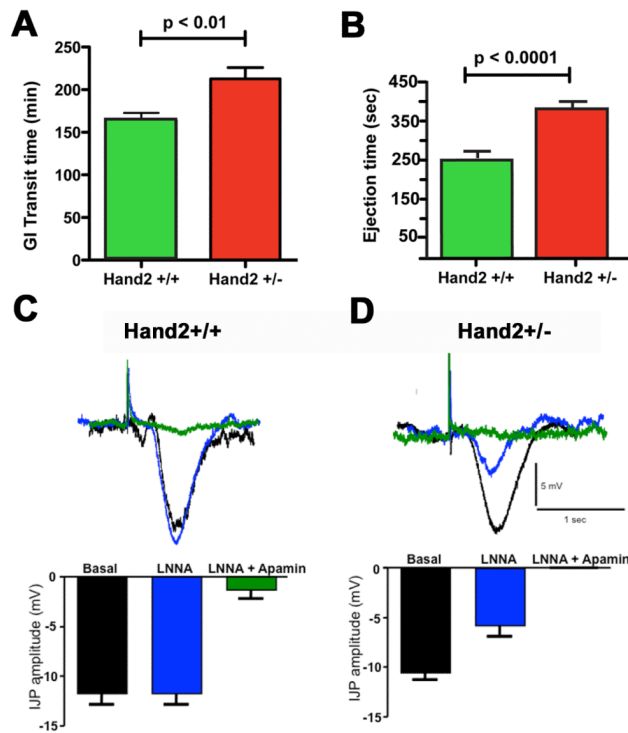


Figure 6. *Hand2* haploinsufficiency slows total gastrointestinal transit and colonic motility
 (A) Total gastrointestinal transit time in *Hand2*^{+/-} mice is significantly greater than that of their *Hand2*^{+/+} littermates. (B) Colonic motility in *Hand2*^{+/-} mice is significantly slower than in their *Hand2*^{+/+} littermates. N=4 (*Hand2*^{+/+}), N=4 (*Hand2*^{+/-}). (C, D) The neurochemistry of inhibitory junction potentials from circular muscle in *Hand2*^{+/+} mice is different from that in *Hand2*^{+/-} animals. C. Intracellular recordings (above) and graph (below) demonstrating that in *Hand2*^{+/+} mice, the NOS inhibitor LNNA did not affect the IJP; nevertheless, the IJP was almost completely eliminated by apamin, which inhibits purinergic neuromuscular signals. B. Intracellular recordings (above) and graph (below) demonstrating that LNNA significantly reduced the amplitude of the IJP in *Hand2*^{+/-} mice and the IJP was eliminated in the presence of LNNA plus apamin. Under basal conditions, the amplitude of the IJP was approximately the same in *Hand2*^{+/+} and ^{+/-} mice. * indicates p<0.01 vs basal; ** indicates p<0.0001 vs basal

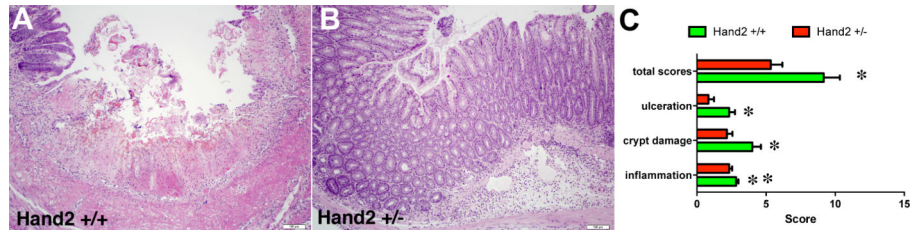


Figure 7. Histological signs of inflammation are greater in *Hand2*^{+/+} than *Hand2*^{+/-} mice
 TNBS was used to induce colitis in *Hand2*^{+/+} and *Hand2*^{+/-} mice. The colons were fixed 7 days later for histological evaluation. A. *Hand2*^{+/+} colon. Note the presence of mucosal ulceration and infiltration of the underlying tissue with leukocytes. B. *Hand2*^{+/-} colon. Ulceration is absent and only moderate edema and leukocyte infiltration is evident. C. The severity of inflammation-induced tissue damage was quantified by assignment of histological scores on a scale of zero to three. The total score and the component scores for ulceration, crypt damage and leukocyte infiltration are illustrated. Total scores and each of the component scores were significantly higher in *Hand2*^{+/+} (green) than in *Hand2*^{+/-} mice (red). * p < 0.01; ** p < 0.05.