Development 138, 4789-4800 (2011) doi:10.1242/dev.060053

Targeted deletion of Hand2 in enteric neural precursor cells affects its functions in neurogenesis, neurotransmitter specification and gangliogenesis, causing functional aganglionosis

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SUMMARY

Targeted deletion of the bHLH DNA-binding protein *Hand2* in the neural crest, impacts development of the enteric nervous system (ENS), possibly by regulating the transition from neural precursor cell to neuron. We tested this hypothesis by targeting *Hand2* deletion in nestin-expressing neural precursor (NEP) cells. The mutant mice showed abnormal ENS development, resulting in lethal neurogenic pseudo-obstruction. Neurogenesis of neurons derived from NEP cells identified a second nestin non-expressing neural precursor (NNEP) cell in the ENS. There was substantial compensation for the loss of neurons derived from the NEP pool by the NNEP pool but this was insufficient to abrogate the negative impact of *Hand2* deletion. Hand2-mediated regulation of proliferation affected both neural precursor and neuron numbers. Differentiation of glial cells derived from the NEP cells was significantly decreased with no compensation from the NNEP pool of cells. Our data indicate differential developmental potential of NEPs and NNEPs; NNEPs preferentially differentiate as neurons, whereas NEPs give rise to both neurons and glial cells. Deletion of *Hand2* also resulted in complete loss of NOS and VIP and a significant decrease in expression of choline acetyltransferase and calretinin, demonstrating a role for Hand2 in neurotransmitter specification and/or expression. Loss of *Hand2* resulted in a marked disruption of the developing neural network, exemplified by lack of a myenteric plexus and extensive overgrowth of fibers. Thus, *Hand2* is essential for neurogenesis, neurotransmitter specification and neural network patterning in the developing ENS.

KEY WORDS: Hand2, ENS, Neurogenesis, Gliogenesis, Neural precursor cell, Neurotransmitter specification

INTRODUCTION

The enteric nervous system (ENS) comprises a network of neural crest-derived neurons that express a diverse array of neurotransmitters and neuropeptides. Although neurons share a large number of phenotypic characteristics, their diversity of connectivity, and neurotransmitter and neuropeptide expression determines their precise function. In neural progenitor cells, the molecular mechanisms responsible for lineage choice, differentiation and acquisition of cell type-specific characteristics are relatively obscure. In the ENS, a diversity of neuronal subtypes and generation of a complicated neural network assures the appropriate pattern of neural connections that drive peristalsis and other coordinated functions of the ENS. Although the molecular signals that direct the generation of neuronal subtype identity in the ENS are not known, the types of neurons and their patterns of connectivity have been described (Hao and Young, 2009). Neurons that make up a single ganglion are not clonally derived, making it difficult to identify the mechanisms responsible for fate specification and differentiation of cell types that support the spatial and temporal developmental profile, but suggesting interplay between intrinsic cues and local extrinsic signaling. The role of proliferation in colonization of the bowel wall by neural crest-derived cells and identification of extrinsic signals and transcriptional regulators that affect differentiation has provided insight into how neuron phenotype diversity is generated in the developing ENS. Proliferation of neural precursor cells is tied to lineage segregation and differentiation by maintaining multipotent cells responsive to local instructive signals as they migrate along the gut tube. The factors that direct enteric neural crest cells to cease their migration and initiate differentiation are not yet known, but the temporal pattern of neurotransmitter molecule expression suggests that localized changes in proliferative state and migration correlates with changes in gene expression that are coincident with differentiation.

Formation of the ENS requires proliferation of neural crest-derived cells as they migrate to colonize the bowel wall. There also is a requirement for sufficient numbers of cells to establish migration in the foregut (Peters-van der Sanden et al., 1993; Burns and Le Douarin, 2000; Burns et al., 2009; Zhang et al., 2010). Cells proliferate as they move along the gut tube and at the migration wave-front; such proliferation is required for complete colonization of the gut wall (Druckenbrod and Epstein, 2007; Simpson et al., 2007; Simpson et al., 2006; Druckenbrod and Epstein 2005; Young et al., 2004; Barlow et al., 2008; Walters et al., 2010). How precursor cell numbers are maintained is unclear. Vagal level neural crest cells have an inherently higher proliferative capacity than do trunk level cells (Zhang et al., 2010), potentially augmenting interactions with environmental signals.

The most important extrinsic signaling molecule for migration, proliferation and survival of enteric precursor cells is glial cell line-derived neurotrophic factor (GDNF). GDNF binds to and activates

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Accepted 23 August 2011

the receptor tyrosine kinase RET, which initiates these processes (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enemoto et al., 1998; Hearn et al., 1998; Young et al., 2001; Natarajan et al., 2002; Wang et al., 2010). Recent studies suggest that patterning connectivity within the ENS neural network depends crucially on Ret-mediated GDNF signaling (Wang et al., 2010), indicating that both cell extrinsic and intrinsic signaling contributes to control proliferation, migration and pattern formation in the developing ENS. Interestingly, GDNF does not affect neuron numbers and cell type-specific marker expression equivalently; precursor cells withdraw from the cell cycle at different times during ENS development in response to varying levels of local GDNF (Wang et al., 2010). Thus, ENS precursor cells may become differentially specified either as they enter the gut wall or during their migration. This implies that differential expression patterns of cohorts of transcriptional regulators control neurotransmitter specification and/or neurotransmitter expression.

Because neural precursor cells continue to proliferate as they migrate, even after they have acquired aspects of pan-neuronal gene expression, we focused attention on the basic helix-loop-helix (bHLH) DNA-binding protein Hand2 as a likely candidate that regulates aspects of neurotransmitter/neuropeptide diversity because it functions in neurogenesis (Baetge et al., 1990; Hendershot et al., 2007; Hendershot et al., 2008; Schmidt et al., 2009; Morikawa et al., 2007) and in neurotransmitter specification in the ENS (Wu and Howard, 2002; Hendershot et al., 2007; D'Autréaux et al., 2007). Targeted deletion of *Hand2* in the neural crest (Danielian et al., 1998; Jiang et al., 2000; Brewer et al., 2004; Hendershot et al., 2007; D'Antréaux et al., 2007) causes significant loss of enteric neurons and loss of vasoactive intestinal polypeptide (VIP) (Hendershot et al., 2007; D'Autréaux et al., 2007), indicating multiple functions for *Hand2* in ENS development. Here, we ask whether Hand2 has a general function in neurotransmitter choice and expression or whether there are subsets of enteric neurons in which Hand2 regulates neurotransmitter/neuropeptide specification. We targeted *Hand2* deletion in neural precursor cells using a nestin-Cre driver line of mice; expression of Cre-recombinase is under control of a neural precursor-specific enhancer.

Our results demonstrate that Hand2 functions in neurogenesis by affecting proliferation of neural precursor cells. Specification and expression of some neurotransmitters/neuropeptides is regulated by Hand2. Patterning of the neural network is impacted by loss of *Hand2*. Furthermore, there appear to be two types of neural precursor cells in the developing ENS, both of which depend upon *Hand2*, but which are segregated based on expression of nestin.

MATERIALS AND METHODS

Targeting strategy

All breeding procedures, animal care and experimental protocols were approved by the UT HSC animal care and use committee. The strategy for targeting *Hand2* has been published previously (Hendershot et al., 2007). Targeted deletion of *Hand2* in neural precursor cells using a Nestin-Cre driver line of mice (The Jackson Laboratory, stock number 003771) is lethal at around P20, allowing analysis throughout development and the early neonatal period. In these mice, nestin expression is regulated by a lineage-specific enhancer located in the second intron midbrain enhancer, thus driving neural precursor-specific deletion (Zimmerman et al., 1994; Yaworsky and Kappen, 1999; Tronche et al., 1999). To generate reporter mice, homozygous R26RYFP females were mated to hemizygous nestin-Cre males and $Hand2^{wt/del;Nestin\text{-}Cre}$ males were mated to $Hand2^{fl/fl;R26R/YFP}$ females.

Expression of transcript encoding *Hand2*

Expression of transcripts encoding *Hand2* was determined by in situ hybridization or by qRT-PCR according to our previously published methods (Wu and Howard, 2002; Zhou et al., 2004; Ruest et al., 2004; Liu et al., 2005; Hendershot et al., 2007).

Immunocytochemistry

Immunostaining was carried out according to our established procedures (Wu and Howard, 2002; Hendershot et al., 2007; Holler et al., 2010). Primary and secondary antibodies used in these studies are listed in Table 1.

Confocal microscopy

Confocal images were acquired using a Leica Microsystems multiphoton confocal microscope (TCS SP5) coupled to a DMI 6000CS inverted microscope, equipped with a Chameleion XR tunable pulsed IR laser and multiple continuous wave lasers. Confocal *z*-stack images were captured using either a 20× objective (n.a.=0.70) or 40× (oil immersion) objective

Table 1. Primary and secondary antibodies used in the reported studies

Antigen detected by primary antibody	Dilution	Source
BLBP (rabbit)	1:500	Chemicon (Temecula, CA, USA)
Calbindine (rabbit)	1:200	Chemicon
Calretinine (goat)	1:200	Chemicon
ChAT (goat)	1:100	Chemicon
Galanin (rabbit)	1:100	Chemicon
GFP (chicken)	1:300	Aves Labs (Tigard, OR, USA)
Hu (human)	1:25,000	A generous gift from Miles Epstein (University of Wisconsin, Madison, WI, USA)
Ki67 (rabbit)	1:300	Abcam (Cambridge, MA, USA)
Nestin (rabbit)	1:100	Chemicon
nNos (rabbit)	1:2000	Chemicon
NPY (rabbit)	1:100	Immunostar (Hudson, WI, USA)
Phox2b (rabbit)	1:800	A generous gift from Françoise Brunet (CNRS, France)
Subtance P (rabbit)	1:500	Chemicon
Tachykinin (rabbit)	1:300	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
TH (rabbit)	1:200	Pel Freez (Rogers, AK, USA)
VachT (rabbit)	1:200	Synaptic System (Göttingen, Germany)
VIP (rabbit)	1:300	Immunostar
Secondary antibody	**Dilution**	**Source**
Anti-chicken FITC	1:100	Jackson ImmunoResearch (Bar Harbor, ME, USA)
Anti-human TRITC	1:200	Jackson ImmunoResearch
Anti-goat Alexa Fluor 647	1:500	Invitrogen (Carlsbad, CA, USA)
Anti-rabbit Alexa Fluor 647	1:500	Invitrogen

(n.a.=1.25) with 4× software magnification at 1.0 mm (whole-mount) or 0.5 mm (tissue sections) steps. To acquire images, fluorescent FITC-coupled, TRITC-coupled and Alexa Fluor 647-coupled secondary antibodies were excited using argon, HeNE or 633 laser lines, respectively.

Cell counts

Cells were counted (field of view) from confocal image stacks using Image J (v.1.42d, http://rsbweb.nih.gov/ij/). Cells expressing individual markers were first visually identified in each optical section and then marked to prevent duplication in counts of cells visible in more than one optical slice. Cumulative counts were compiled from the entire stack for each image. Background subtraction was performed on full stacks using the built-in algorithm with a radius of 50. The ROI manager utility was used to save the coordinates of the marked cells; this allowed determination of which cells co-expressed different markers. To determine co-expression (e.g. Hu and YFP), cells expressing one label were identified and scored. The ROIs were saved and then overlaid onto the images of the second label. Each identified cell was scored as either positive (+) or negative (–) for the co-label. To determine co-expression of three labels (e.g. YFP, Hu, Ki67), imagers of two of the markers (e.g. YFP, Hu) were first identified, marked and merged; the resulting ROI set was then overlaid on the third image stack and counted as described above. Cells were counted in comparable areas from a minimum of three animals for each condition.

Statistics

Data are presented as the mean±s.e.m. unless otherwise stated. Statistical significance was determined using Student's unpaired *t*-test or ANOVA and Bonferroni post-hoc test, unless otherwise stated.

RESULTS

Deletion of Hand2 is coincident with nestin expression

Targeted *Hand2* deletion in the neural crest decreases neurons in the ENS (Hendershot et al., 2007; D'Autrèaux et al., 2007). To understand better the mechanism that underlies this effect on neurogenesis, we targeted *Hand2* deletion in neural precursor cells (Zimmerman et al., 1994; Wiese et al., 2004; Mignone et al., 2004; Dubois et al., 2006). Cells expressing nestin in neural precursor cells were genetically marked and visualized by expression of YFP. We confirmed the efficacy of targeted *Hand2* deletion by counting the number of neural crest-derived cells (p75) that express the YFP reporter. At E12.5 in small intestine, 86% of $p75^+$ cells also express YFP; by E14.5, this number increases to 90% in small intestine and to 96% in colon (Young et al., 1999). Using immunocytochemistry, we verified that expression of nestin protein and Cre overlap in neural precursor cells (data not shown) and that YFP and nestin also overlap (Fig. 1).

Targeted *Hand2* deletion using nestin-Cre caused staggered deletion of *Hand2* in neural precursor cells as expression of nestin is established and increases with developmental time (Fig. 1). At E10.5, nestin expression is detectable in the small intestine (Fig. 1A) and colon (Fig. 1E) but is confined to non-neural cell types (white arrows). Expression of YFP is undetectable. By E12.5, cells expressing nestin immunoreactivity (red) and YFP reporter (green) are visible in developing myenteric plexus (blue arrows) and within the colon wall. Nestin expression is well established by E14.5 within the developing myenteric plexus (Fig. 1C,G). Nestin expression in neural precursor cells, endothelial cells and other non-neuronal cells, is maintained until E18.5, the last time-point analyzed. Thus, by E14.5 expression of transcripts encoding *Hand2* should be substantially reduced. Based on qRT-PCR (Fig, 2E) and in situ hybridization (Fig. 2A-D), expression of transcripts encoding *Hand2* is reduced by 61% ($P<0.01$) at E14.5. By E18.5, expression of transcripts encoding *Hand2* is reduced by 78% ($P<0.001$); based on cell counts of $p75^+$/GFP- and Hu^+/nestin-expressing cells (E12.5), ~15% of cells comprise a nestin non-expressing (NNEP) pool that accounts for the *Hand2* expression not affected by deletion in the nestin-expressing (NEP) pool. At E14, there is no apparent effect on embryo growth by *Hand2* deletion in neural precursor cells; wild-type and mutant embryos were found consistently to be of the same size and had normal gross morphology (Fig. 2F). Expression of nestin, based on YFP reporter localization, coincides with reported patterns (for reviews, see Wiese et al., 2004; Kawaguchi et al., 2001; Dubois et al., 2006): expression of nestin was not detected in vagal neural crest-derived cells destined to colonize the gut wall (Fig. 2G). *Hand2*

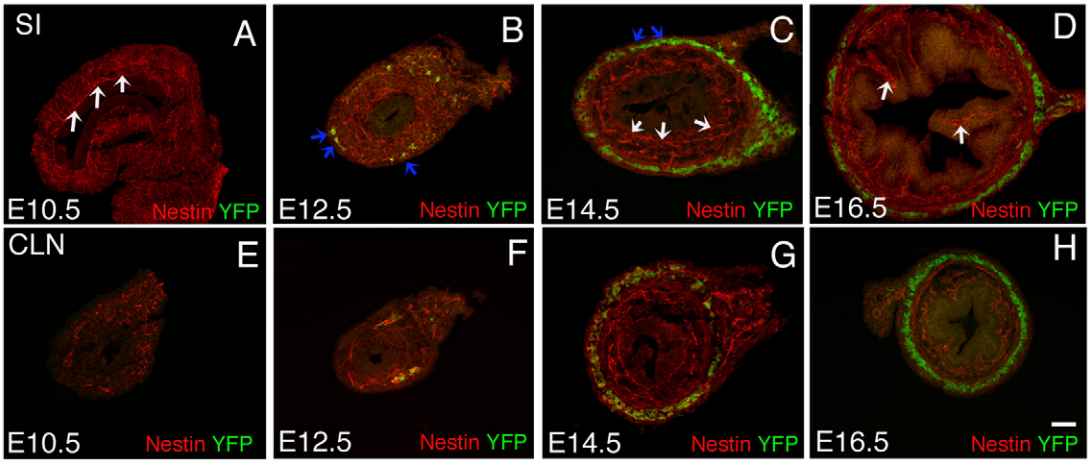

Fig. 1. Nestin expression profile. (**A-H**) Immunocytochemistry was used to demonstrate the developmental expression profile of nestin protein (red) and reporter (YFP, green). Beginning at E10.5, in small intestine (SI), nestin protein was expressed in non-neural cells (white arrows in A, C and D) but YFP was not detected in these cells because the nestin driver is neuron precursor specific. Expression of YFP was first detected at E12.5 in myenteric ganglia (blue arrows). From E12.5, the number of cells expressing both nestin and nestin reporter increased with advancing developmental age. Nestin was rarely detected in submucosal ganglia. Expression of nestin reporter overlapped completely with nestin expressed in neurons, indicating that *Hand2* would be deleted in all neural nestin-expressing cells. Images were photographed at 10× magnification. Scale bar: 50 μm.

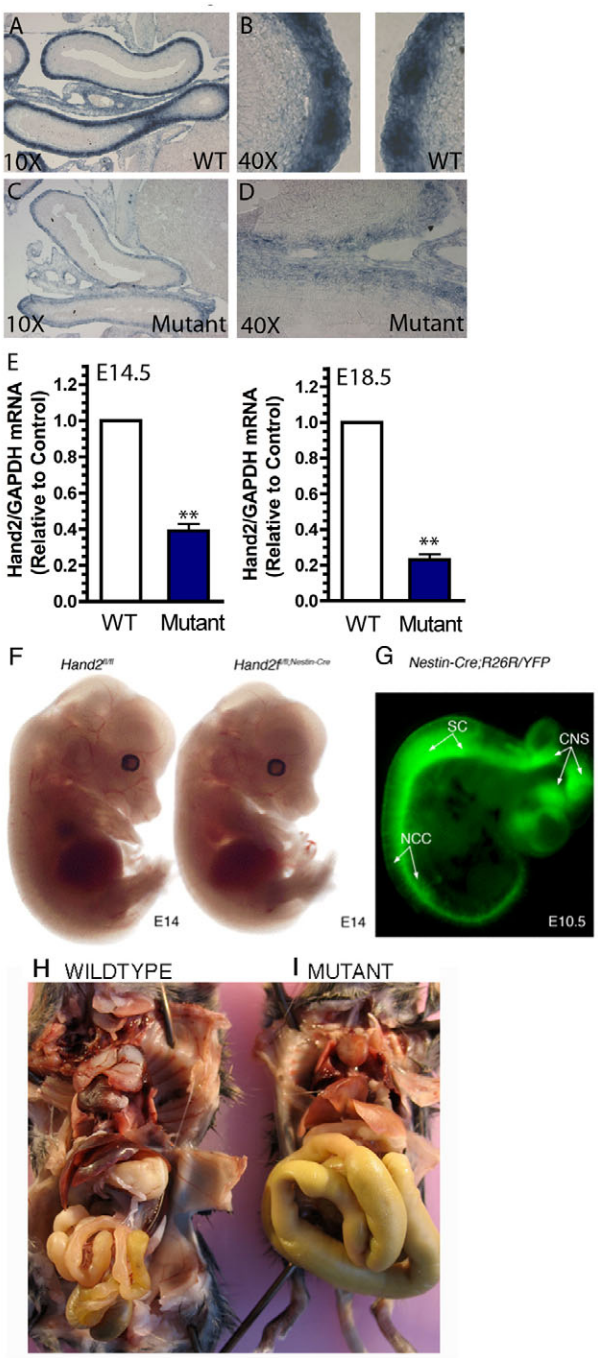

Fig. 2. *Hand2* is deleted significantly in *Hand2*$^{fl/del;Nestin\text{-}Cre}$ embryos. (**A-D**) In situ hybridization (E14.5), used to visualize transcripts encoding *Hand2,* showed a substantial decrease in the number of mutant proximal gut myenteric ganglia (C,D) when compared with wild type (A,B). (**E**) qRT-PCR confirmed a significant decrease of transcripts encoding *Hand2* that became more pronounced with increasing developmental age. Data are mean±s.e.m. $^{**}P<0.001$. (**F**) The size and overall appearance of E14 *Hand2*$^{fl/fl}$ embryos compared with *Hand2*$^{fl/del;Nestin\text{-}Cre}$ embryos was comparable. (**G**) Nestin reporter is expressed in the CNS and a subpopulation of neural crest (NCC); there was no apparent nestin expression in the vagal neural crest or forming gastrointestinal tract. SC, sympathetic chain. (**H**,**I**) Although at early stages of development, overall embryo growth was not affected by deletion of *Hand2*, by P20, mutant mice were significantly smaller than wild-type mice and had a severely distended (I) bowel compared with wild type (H).

deletion in neural precursor cells is not embryonic lethal, although mutant mice die around P20 (Fig. 2H,I). By P20, mutant mice lag in growth and are smaller than wild-type littermates. The cause of death is unknown but distention of the gastrointestinal tract may be a contributing factor (Fig. 2I). To understand the underlying basis for the ENS defect that eventually proves lethal, we examined development of the ENS beginning at E12.5.

Hand2 deletion negatively impacts neurogenesis and reveals two different neural precursor cells

At E12.5, there was a significant ($P<0.001$) reduction in the number of neurons (193±24 versus 35±4) that developed in the absence of *Hand2* (Fig. 3, hatched bars); cells were identified based on expression of YFP and the pan-neuronal marker HuC/D (Furness et al., 2004; Qu et al., 2008). The total number of neurons increased with developmental age, but there remained a significant ($P<0.001$) decrease in Hu$^+$/YFP-expressing neurons at E14.5 (552±23 versus 223±19) and E18.5 (666±41 versus 278±44) in the ENS of *Hand2* mutant embryos. Interestingly, all neurons developing in the gut wall were not derived from NEP cells; neurons were also derived from NNEP cells. In the absence of *Hand2*, there was a significant ($P<0.001$) increase in the number of neurons derived from nestin-negative precursor cells (Fig. 3, white bars). Throughout embryonic development, the number of Hu$^+$/YFP$^-$ neurons increased in both wild-type and mutant embryos, with a significantly larger number developing in the mutant embryos. At E12.5 there was no significant difference in the total number of neurons that developed in the wild-type compared with mutant ENS (239±19 versus 177±30). Whereas in the wild-type gut, 80±4% of neurons developed from NEP cells, only 21±5% of neurons in the mutant developed from these precursor cells. At E12.5, 80% of cells expressing Phox2b also express YFP, and of these cells, 77% also express Hu. In the mutant, 43% of Phox2b$^+$ cells also express YFP and, of these cells, 34% also express Hu. By E18.5, 91±2% of neurons developed from NEP cells in wild-type ENS, whereas 45% of neurons in the mutant developed from this precursor cell. At each age examined, significantly fewer NEP cells differentiate as neurons in the mutant. The major increase in neuron number occurred between E12.5 and E14.5. The decrease in NEP-derived neurons and the compensatory increase in NNEP-derived neurons in *Hand2* mutant ENS prompted examination of glial cell development (Fig. 4); we examined whether *Hand2* affects glial cell number and whether there was differential contribution or compensation from the two pools of neural precursor cells.

Glial cell (BLBP IR) and neuronal development were examined at P20 to prevent potential problems with timing of appearance of glial cell markers. The significant decrease in NEP-derived neurons observed in the embryonic ENS was maintained in *Hand2* mutant neonates (Fig. 4A). In the small intestine, 35±3% of neurons in the mutant and 74±2% in the wild-type were derived from NEP cells. Neuronal development in the colon (Fig. 4A) followed the same pattern. As there are two populations of glial cells in the ENS, one in the ganglia and one associated with nerve fibers, we analyzed them separately (Fig. 4B,C). In small intestine myenteric ganglia, there was a significant ($P<0.01$) decrease in the number of glial cells derived from the NEP (Fig. 4B, hatched bars). There was no difference in the number of glial cells derived from the nestin-independent population (Fig. 4B, white bars). In the mutant, there was a significant decrease in glial cells derived from the NEP associated with nerve fibers, but there was no significant effect on those cells derived from the NNEP. In the colon, there was no

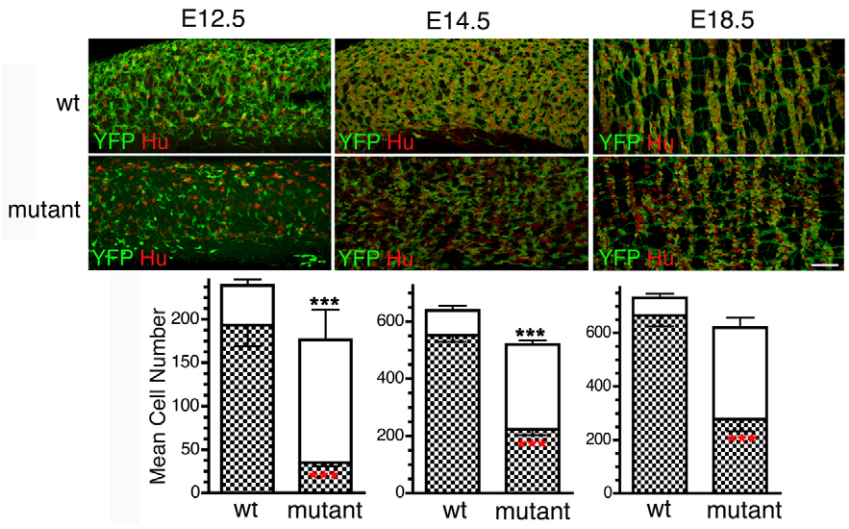

Fig. 3. Deletion of *Hand2* reveals two types of neural precursor and produces a significant change in neurogenesis. In small intestine there was a significant decrease in neurogenesis from NEP (hatched bars, ***P<0.0001) cells (Hu^+/YFP^+) throughout development. The increased neurogenesis from NNEP (white bars, ***P<0.0001) cells (Hu^+/YFP^-) in the *Hand2* mutant compensated for the significant loss of neurons from the NEP pool of cells. Neurons were identified and counted based on immunoreactivity to the HuC/D antigen. Compensation in neural numbers by NNEP-derived cells was not sufficient to prevent development of an abnormal ENS. Data are mean±s.e.m. Scale bar: 50 μm.

significant difference between mutant and wild-type glial cell development in the ganglia, but there was a significant (P<0.01) decrease in the number of glial cells associated with nerve fibers derived from NEP cells (Fig. 4C). It appears that glial cells associated with both nerve fibers and myenteric ganglia are primarily derived from the NEP pool of cells. We substantiated that *Hand2* would be deleted in both neural precursor cells and glial cells by determining that 91% of cells expressing Sox2 also express YFP; Sox2 is a good marker for ENS progenitors and glial cells (Heanue and Pachnis, 2011).

Hand2 affects proliferation of neural precursor cells

Previously, we reported that Hand2 affects cell proliferation (Hendershot et al., 2008; Holler et al., 2010; Barron et al., 2011). The significant decrease observed in both neuron and glial cell numbers in *Hand2* mutant ENS suggested that loss of *Hand2* function in the NEP cells might cause this anomaly. The number of proliferating neuroblasts (Ki67 expressing) was determined at E12.5 in small intestine (Fig. 5A) and at E14.5 in small intestine (Fig. 5B) and colon (Fig. 5C). At E12.5, there was a significant (P<0.001) decrease in the number of NEP-derived neuroblasts/neurons in mutant small intestine (35±4) compared with wild type (193±24). Of proliferating neuroblasts, 86% were derived from the NEP pool in the wild type compared with 22% in the mutant. This was compensated for by a significant (P<0.001) increase in the number of neurons in the mutant (78%) derived from the NNEP pool of cells compared with the control (14%). At E14.5, the pattern in the small intestine is similar to that observed at E12.5 but the percentage of proliferating cells in the NNEP pool is reduced to 65% in the mutant. At E14.5, in the colon (Fig. 5C), there is a significant (P<0.01) decrease in the number of proliferating neuroblasts derived from NEP cells (114±17 versus 25±3); only 24% of cells were proliferating in the *Hand2* mutant compared with 47% of cells in control mice. There was a significant increase in the number of proliferating cells derived from the NNEP pool of cells in the mutant (38%) compared with wild-type (7%) gut. The overall degree of compensation in the colon was smaller than in the small intestine. There was no apparent cell death in either wild-type or *Hand2* mutant ENS, suggesting the difference in cell numbers was due primarily to changes in the proliferative capacity of enteric neural precursor cells and neuroblasts.

Myenteric ganglia do not form in the absence of Hand2

Although there are sufficient cells to support migration throughout the developing gut wall, in the absence of *Hand2*, few, if any, discrete ganglia form in either the small intestine (Fig. 6) or colon (Fig. 7); the patterning defect is apparent in the embryonic gut but becomes increasingly pronounced as development progresses. By P20 (Figs 6, 7) this defect worsens from the proximal to distal bowel wall and is not confined to the neurons. In the small intestine (Fig. 6), glial cells are located in both the myenteric ganglia and along the fiber tracts (Fig. 6D,H). In the mutant, however, few glial cells associated with fibers, and there were many neurons with no associated glial cells (Fig. 6L,P). In the colon (Fig. 7), there are no well-defined ganglia and there is an extensive overgrowth of disorganized fibers (Fig. 7L,P); however, glial cells remained associated with the neurons.

Neurotransmitter/neuromodulator specification and expression is affected by Hand2 function

In the mouse ENS, there are 11 subtypes of myenteric neurons and five subtypes of submucosal neurons (Sang and Young, 1996; Young et al., 2003; Qu et al., 2008). We examined expression of neurotransmitters and neuromodulators to distinguish the various classes of neurons: we looked at choline acetyltransferase- (ChAT), nitric oxide synthase- (NOS), calbindin- (calB), calretinin- (calR), vasoactive intestinal polypeptide- (VIP), substance P- (SP), NPY- and galanin-containing neurons.

In chicken (Hendershot et al., 2007) and mouse (Hendershot et al., 2007; D'Autrèaux et al., 2007) ENS, loss of *Hand2* function in the neural crest results in absence of VIP-expressing neurons. In cells where deletion of *Hand2* is targeted to the NEP cells, expression of VIP is absent (data not shown). This comprehensive loss of VIP suggested that other neurotransmitters/neuromodulators would be affected similarly; this is important because diversity of neurotransmitters/neuromodulators impacts the functional architecture of the ENS. Our analysis showed that Hand2 does not impact expression of all neurotransmitters and neuromodulators in the ENS. The expression of calB was not affected by loss of *Hand2* in NEP cells (Table 2), and was significantly (P<0.05) increased in the neuron population derived from the NNEP in *Hand2* mutant ENS. In wild-type ENS, calB neurons are derived primarily from the NEP cells. There were significant decreases in the numbers of

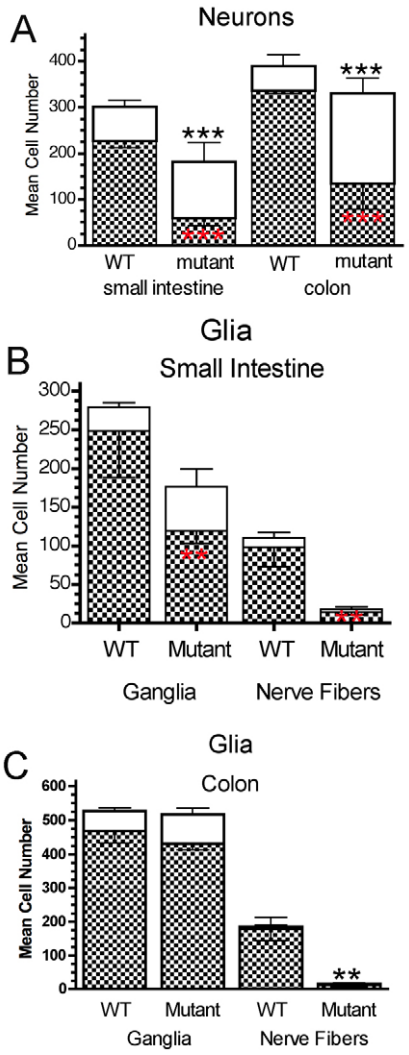

Fig. 4. Gliogenesis is negatively affected by deletion of *Hand2*. (**A**) At P20 there was a significant decrease in the number of neurons (Hu^+ cells) that developed from NEP cells (hatched bars); there was significant compensation from NNEP cells (white bars). (**B**,**C**) Glial cells (counted and identified by expression of BLBP) in the myenteric ganglia in the small intestine (B) and colon (C) are derived primarily from NEP cells (hatched bars) with no compensation by NNEP cells. In the colon (C), there was no significant decrease in the number of glial cells in the myenteric ganglia of *Hand2* mutant embryos. There was a significant decrease in glial cells associated with nerve fibers. Enteric glial cells are primarily derived from a *Hand2*-dependent NEP cell with little compensation for their loss, indicating differential regulation of the glial lineage compared with the neuronal lineage. Data are mean±s.e.m. ***P*<0.001; ****P*<0.0001.

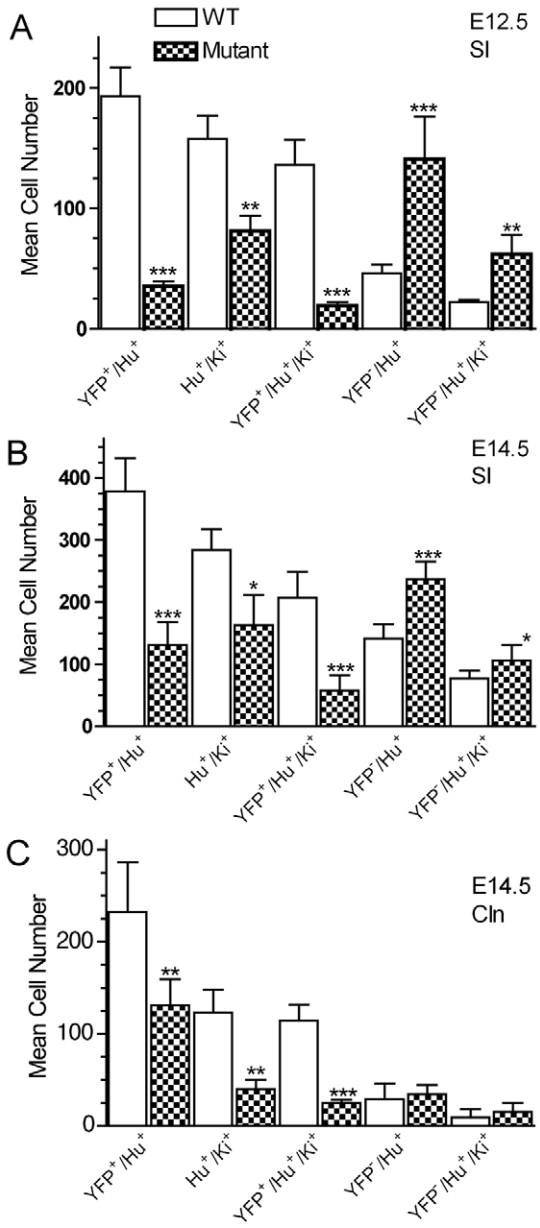

Fig. 5. NEP cells have a different proliferation potential from NNEP cells. (**A-C**) The number of cells expressing YFP (nestin reporter), Hu (neuron marker) and/or Ki67 (proliferating cells) was counted at E12.5 (A) and E14.5 (B,C) in $Hand2^{wt/wt;Nestin\text{-}CreR26RYFP}$ (white bars) and $Hand^{fl/del;Nestin\text{-}Cre/R26RYFP}$ (hatched bars) embryonic small intestine (A,B) and colon (C). At both ages, there was a significant decrease in the number of neurons derived from the NEP pool of cells; these cells are dependent on *Hand2* for proliferation. The number of neuroblasts proliferating was also significantly decreased by deletion of *Hand2*. There was compensation at E12.5 (A) in the NNEP pool of cells, which showed significant increases in the number of proliferating neuroblasts in the *Hand2* mutant (hatched bars). In the colon (C), although neurogenesis was significantly decreased in the *Hand2* mutant, proliferation of the NNEP cells was low. In both the control and the mutant, 70-80% of Hu^- cells were dividing at E12.5 and E14.5. Data are mean±s.e.m. **P*<0.01; ***P*<0.001; ****P*<0.0001.

neurons expressing ChAT, NOS or calR in neurons derived from NEP cells in mutant small intestine and colon (Table 2) compared with wild-type ENS. Although there was a significant ($P<0.001$) increase in the number of neurons expressing ChAT in cells derived from NNEPs in the mutant, no such compensatory increase was observed for either NOS or calR. For neuromodulators that are primarily detectable in fibers (data not shown), deletion of *Hand2* in NEP cells abrogated NYP expression in the colon. In the small intestine, it is associated mainly with the NNEP cell population. Tachykinin was associated primarily with NEP-derived neurons in wild-type ENS and was almost completely lost in small intestine. It was associated with the NNEP-derived neurons in the colon. Using an anti-substance P antibody, we detected only a slight decrease in expression when comparing wild-type with mutant gut

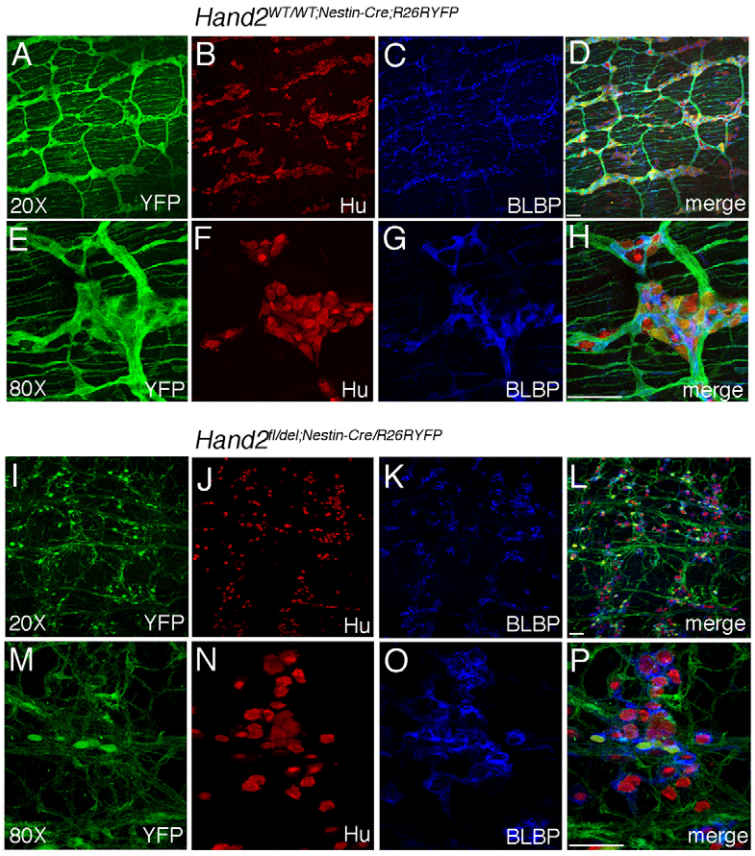

Fig. 6. Myenteric ganglia do not form in small intestine when *Hand2* is deleted in NEP cells. Immunocytochemistry was used to examine patterning of the myenteric plexus at P20. Expression of nestin (YFP, green), Hu (red) and BLBP (blue) was analyzed in confocal images shown as *z*-stack projections. (**A-H**) In wild-type gut, ganglia formed in the lattice pattern typical of the ENS. Glial cells (C,D,G,H) were localized in the myenteric ganglia around the neurons and in the connective tissue along processes (D,H). The majority of neurons were derived from the NEP (A,E) cells but there were some ganglia (H) where there was substantial contribution from NNEP-derived cells (red cells that are not also green). (**I-P**) The deletion of *Hand2* in the NEP cells resulted in a disorganized collection of cells that rarely formed ganglia. The disorganized pattern of cells is easily recognizable in the nestin expressing cells (I,M). There is no lattice pattern visible in either the neurons (J,N) or the glial cells (K,O). The merged images (L,P) show that fiber tracts fail to form and that the fibers are disorganized. There are few glial cells (K,O) associated with nerve fibers but there are glial cells associated with the neurons (L,P). Scale bars: 50 μm.

where *Hand2* deletion was targeted in the NEP cells. In the *Hand2* mutant small intestine, expression of NPY was associated with the neurons derived from the NNEP cells; expression of NPY was not observed in the colon of mutant mice. NPY was associated mainly with neurons derived from the NEP cells in wild-type animals. The expression of galanin was not affected by deletion of *Hand2*. Thus, neurotransmitter/neuromodulator specification is determined independently of other aspects of neurogenesis and Hand2 does not serve some universal function in this aspect of phenotype determination.

Hand2 is expressed in both types of neural precursor cell

Our results indicate the existence of two populations of neural precursor cells; one expresses nestin and one does not. The NEP cells give rise to both neurons and glial cells. The glial cells, however, are derived primarily from NEP cells; neurons are derived from both NEP and NNEP. We therefore asked whether each of these neural precursor cells was dependent upon Hand2 for aspects of neurogenesis and cell type-specific gene expression (Fig. 8). *Hand2* was expressed in all glial cells derived from the NEP pool. *Hand2* was not expressed in glial cells that did not express YFP. There was a small population of neurons derived from the NNEP cells (Fig. 8C,F, arrows) that do not express transcripts encoding *Hand2*. Thus, there may be two neural precursor cells in the ENS, both of which express *Hand2*. The NNEP cells are biased towards differentiating as neurons and appear to have a different potential for proliferation than the NEP cells. This conclusion is borne out by examination of the ratio of glial cells to neurons at P20. The glial cell to neuron ratio is 1.09 for control and 2.02 for mutant cells derived from the NEP pool. Cells derived from the NNEP pool differentiate in the same ratio as the NEP pool in control embryos but in the knockout the ratio drops to 0.46, indicating preferential differentiation as neurons.

DISCUSSION

Our goal was to elucidate the consequences to ENS development of targeted deletion of *Hand2* in neural precursor cells. From previous studies (Hendershot et al., 2007; D'Autreaux et al., 2007), it was unclear whether *Hand2* was expressed in, and required for, development of enteric glial cells, whether *Hand2* was involved in specification/neurogenesis or differentiation/acquisition of cell type-specific properties, how *Hand2* affected neuron number, and whether *Hand2* determined expression of all neurotransmitters and neuromodulators. Deletion of *Hand2* at a later developmental stage addressed these issues and identified several neural crest-derived neural precursor cells, one of which may be specific to the ENS.

Neural precursor cells in the ENS

The neural crest is exemplified by its developmental potency and plasticity. Although it is accepted that many lineages segregate early and that patterning of the peripheral nervous system is determined essentially by the timing of delamination from the neural tube and which migratory pathway the cells follow (Kasemeier-Kulesa et al., 2005; Kasemeier-Kulesa, 2006; Kulesa et al., 2009a; Kulesa et al., 2009b; Krispin et al., 2010), identification of a 'neural precursor' has not been definitive. Using the nestin neuroepithelial enhancer to drive expression of Cre recombinase to target deletion of *Hand2* we identified two neural

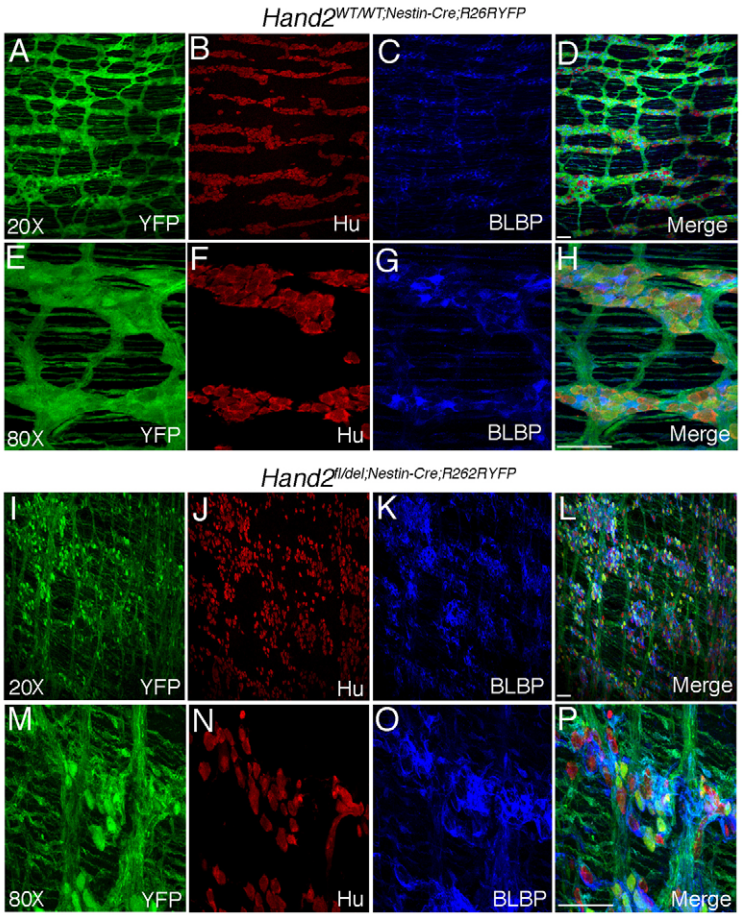

Fig. 7. Myenteric ganglia fail to form in the colon of *Hand2* mutant mice. Immunocytochemistry was used to examine patterning of the myenteric plexus at P20. Expression of nestin (YFP, green), Hu (red) and BLBP (blue) was analyzed in confocal images shown as *z*-stack projections. (**A-P**) A comparison of wild-type (A-H) colon with *Hand2* mutant colon (I-P) shows remarkable disruption of the myenteric plexus. The most striking difference is the very dense, albeit disorganized, fiber network that developed when *Hand2* was deleted in the NEP cells (I,M). The glial cells (K,O) remain associated with the neurons (J,N), although discrete ganglia fail to form. The majority of neurons are derived from the NNEP cells (L,P). Scale bars: 50 μm.

precursor cells. One cell is nestin expressing and gives rise to both neurons and glial cells in the ENS. The second does not express nestin and generates mostly neurons in the ENS. Previous reports (D'Autreaux et al., 2007) indicating that Hand2 does not affect gliogenesis in the ENS differ from what we report here. Differences in the age at analysis, differences in the mouse model, and the fact that cells were examined in explant culture probably account for the fact that no effect on glial cells was observed in the previous studies.

We posit that the specific identity of the neural precursor cells is instructed prior to or at the time of their migration from the neural tube and that their differentiation is directed by the local microenvironment. Neural precursor cells with intrinsic differences that give rise to specific cell types have also been identified in the olfactory epithelium (Murdoch and Roskams, 2008). Transplantation of enteric neural crest-derived cells into the trunk migratory region of a young host results in localization of donor cells in the DRG and SG, but the cells fail to express neuronal phenotypic characteristics for either sensory or noradrenergic neurons (Pisano and Birren, 1999) supporting the idea that the neural precursor cells are intrinsically different and thus have specific developmental potential.

Targeted deletion of Hand2 alters ENCC proliferation

We have previously shown that *Hand2* functions in neurogenesis (Hendershot et al., 2008), development of the cardiac outflow tract (Holler et al., 2010) and aspects of craniofacial morphogenesis (Barron et al., 2011) by regulating cell proliferation. Here, we show a significant decrease in the number of dividing NEP cells when *Hand2* was deleted although there was only a slight decrease in the total number of neurons. The neuronal population in the *Hand2* mutants is differentially composed of more NNEP-derived neurons, demonstrating that a subset of neuroblasts proliferate in the ENS (Young et al., 2005; Hao et al., 2009). In these mice, the nestin-negative population increases their proliferation as a compensatory mechanism to ensure that sufficient numbers of neurons are generated to colonize the entire length of the bowel.

This compensation suggests that there is some form of cell number sensor either within the migratory cells or within the environment that can signal to the cells to alter their proliferation rate. This idea is supported by mathematical modeling demonstrating that increased proliferation could substitute for a decrease in the initial number of cells colonizing the gut (Zhang et al., 2010) and is reminiscent of the differential proliferative capacity of vagal and sacral neural crest-derived cells (Newgreen et al., 1980; Delalande et al., 2008; Zhang et al., 2010). These results show that both the initial number of precursor cells and differential proliferative capacity accounts for neuron numbers in the ENS (Zhang et al., 2010). The mechanism that is being adopted in the *Hand2*-deficient mice is not clear; however, both epidermal growth factor (EGF) and fibroblast growth factor 2 (Fgf2) have been shown to impact the number of progenitors (Fgf2) and the number of enteric neural crest cells formed (EGF) (Fuchs et al., 2009). The intracellular mediator of the activity of EGF is dependent upon the Rho GTPases Cdc42 and Rac1 (Fuchs et al.,

Table 2. Neurotransmitter/neuromodulator expression

Neurotransmitter/neuromodulator	Mean neuron number±s.e.m. Wild type	Mutant	*P* value
Choline acetyltransferase (ChAT)			
Total ChAT neurons small intestine	451±25	386±16	>0.05
Nestin$^+$ neurons	373±22	151±10	<0.001
Nestin$^-$ neurons	60±19	236±7	<0.001
Total ChAT neurons colon	706±64	584±13	>0.05
Nestin$^+$ neurons	669±69	343±17	<0.01
Nestin$^-$ neurons	36±11	241±5	<0.001
Nitric oxide synthase (NOS)			
Total NOS neurons small intestine	268±15	25±10	<0.001
Nestin$^+$ neurons	259±25	12±4	<0.001
Nestin$^-$ neurons	10±9	13±3	>0.05
Total NOS neurons colon	458±28	15±5	<0.001
Nestin$^+$ neurons	447±25	12±3	<0.001
Nestin$^-$ neurons	11±5	2±3	>0.05
Calretinin (CalR)			
Total CalR neurons small intestine	113±16	38±9	<0.01
Nestin$^+$ neurons	90±12	18±5	<0.01
Nestin$^-$ neurons	23±17	20±12	>0.05
Total CalR neurons colon	279±15	171±8	<0.01
Nestin$^+$ neurons	275±15	157±15	<0.01
Nestin$^-$ neurons	4±2	14±6	>0.05
Calbindin (CalB)			
Total CalB neurons small intestine	59±10	116±26	>0.01
Nestin$^+$ neurons	48±6	38±18	>0.05
Nestin$^-$ neurons	11±6	78±18	<0.05
Total CalB neurons colon	27±10	49±3	>0.05
Nestin$^+$ neurons	26±10	23±6	>0.05
Nestin$^-$ neurons	1±0.8	26±6	<0.01

The number of cells expressing each neurotransmitter or neuromodulator was counted in whole-mount segments of small intestine and colon. Whole-mount segments were labeled with antibodies directed against the neurotransmitter (biosynthetic enzyme) or neuromodulator, against Hu to identify neurons, and against YFP to identify cells derived from the nestin-expressing neural precursor pool of cells. Neurons expressing Hu but not expressing YFP were counted as nestin$^-$ neurons. Cells were counted from confocal stacks. Cells were counted in three segments of small intestine and colon from three animals of each genotype.

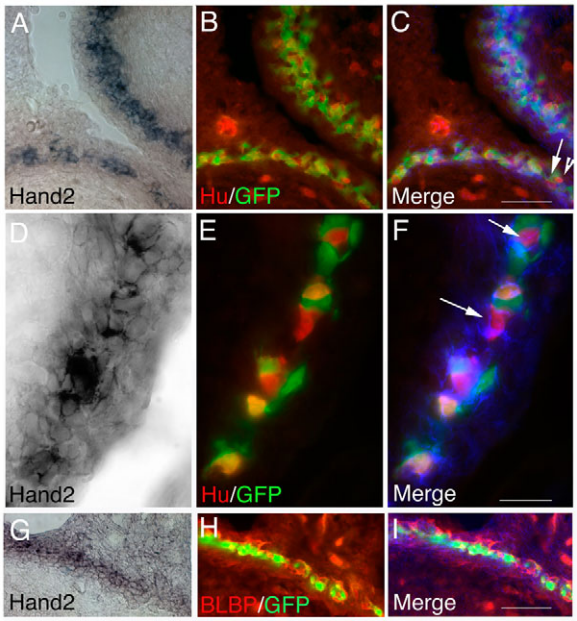

Fig. 8. *Hand2* is expressed in the majority of neural precursor cells. (**A-I**) In situ hybridization was combined with immunocytochemistry to determine the overlap between expression of transcript encoding *Hand2* (A,D,G), the neuronal marker Hu (B,C,E,F) and the glial marker BLBP (H,I). The majority of neurons are NEP-derived cells. (C) A few neurons are derived from NNEP cells that do not express *Hand2* (arrows in C and F). All glial cells are derived from *Hand2*-expressing NEPs. Scale bars: 50 μm.

2009). As we previously identified Cdc42 and Rac1 as direct Hand2 targets (Holler et al., 2010), these genes could be responsible for the defects observed in our mice.

Gangliogenesis does not occur in the absence of Hand2

The significant decrease in NEP cells in *Hand2* mutant mice has a profound effect on patterning of the myenteric plexus. Mathematical modeling suggests that proliferation can affect both migration and plexus formation in the developing ENS (Landman et al., 2007; Binder et al., 2008; Zhang et al., 2010). Based on these models we expected that loss of *Hand2* might result in hypoganglionosis or aganglionosis in the distal bowel or that smaller ganglia might form (Goldstein et al., 2005). Instead, along the entire length of the bowel we observed a very disorganized collection of neurons and support cells that lacked all aspects of the normal lattice-patterned ganglionated neural network characteristic of the myenteric plexus. We also observed increased density of fibers with little organization around cell bodies or between fiber tracts in the *Hand2* mutant. This phenotype could be caused by altered cell-cell interactions (Kapur et al., 1995). Changes in the expression of cell surface molecules could impact adhesion and clustering into ganglia thereby disrupting neural network patterning. The neural adhesion molecule NCAM mediates some aspects of gangliogenesis in the ENS (Faure et al., 2007); NCAM is a direct Hand2 target (Holler et al., 2010) and its expression is significantly reduced when *Hand2* is deleted in stem cell-derived neural precursor cells (M.J.H., unpublished result).

It is well documented that some enteric neural crest cells migrate along leading axon-like processes (Young et al., 2002; Young, 2008; Hao and Young, 2009); some of these cells express pan neuronal markers (Conner et al., 2003; Barlow et al., 2008) and NOS (Hao and Young, 2009) and may provide a roadmap for the movement of specific subsets of cells. Since expression of NOS is essentially absent in the *Hand2* mutant ENS, it is possible that the spatial and temporal pattern of migration is disrupted resulting in a disorganized scattered population of neural crest, neural precursor cells and neurons.

Hand2 influences the chemical coding of the mouse ENS

The ENS can mediate reflex activity independent of the spinal cord and brain. The local motor circuit comprises excitatory and inhibitory motoneurons, sensory neurons, interneurons and intrinsic primary afferent neurons. Formation of the functional motor circuit probably requires clustering of neurons to form ganglia. Death of the *Hand2* mutant mice at around P20 coupled with the distention and disorganized appearance of the neurons suggests that the normal circuitry is not properly formed in the absence of *Hand2*.

We found significant decreases in the number of cholinergic and nitrergic neurons in *Hand2* mutants. As cells expressing two of the major neurotransmitters in the ENS, nitric oxide (NO) and acetylcholine (ACh), are born relatively early (Rothman and

Gershon, 1982; Pham et al., 1991; Hao and Young, 2009) the significant decrease in both of these cells in the progeny of the NEP cells suggests that neurotransmission would also be negatively affected. The complete loss of NOS is of particular interest because of the potential effect of NOS neurons on directing migratory patterns and neurotransmission. NOS is required for the biosynthesis of NO, the primary neurotransmitter of inhibitory muscle motoneurons. NOS is also found in interneurons that innervate both longitudinal and circular muscle (Qu et al., 2008). Although early in development VIP is not yet co-expressed with NOS (Rothman et al., 1984; Hao and Young, 2009), later in development VIP is expressed in ~93% of NOS expressing neurons (Sang and Young, 1996; Sang and Young, 1998; Qu et al., 2008). The almost complete loss of NOS and VIP suggests that the expression of these neuromodulators is co-dependent upon *Hand2* or some Hand2-dependent downstream signal. The physiological consequences to loss of the NOS/VIP neurons appears to manifest in motility dysregulation, probably owing to loss of a major component of inhibitory synaptic transmission thereby drastically reducing smooth muscle relaxation.

Calretinin is expressed in ~52% of enteric neurons (Qu et al., 2008). CalR immunreactivity is found in processes innervating both longitudinal and circular muscle in excitatory muscle motoneurons, interneurons and intrinsic sensory neurons (Qu et al., 2008). Deletion of *Hand2* in the NEP cells resulted in a significant decrease in the number of neurons expressing calR. Interestingly, there was no compensation for this loss in the neurons that developed from NNEP cells. Many neurons expressing calR also express substance P (Qu et al., 2008). Expression of substance P was not affected by deletion of *Hand2*. The fact that expression of *Hand2* is not sufficient for expression of a subset of neuromodulators supports the idea that specification of neurotransmitter/neuromodulator in the ENS can occur independently of specification to the neuronal lineage and occurs in response to cell intrinsic signals. Identification of the co-factors that regulate expression of those molecules where Hand2 is not sufficient remains an interesting area for investigation.

There was a significant compensatory increase in the number of neurons expressing ChAT, the enzyme required for the synthesis of acetylcholine, and calB in cells derived from the NNEP pool when *Hand2* was targeted in NEP cells. Acetylcholine is the primary excitatory (longitudinal and circular muscle) neurotransmitter in the ENS and it is also expressed in ascending interneurons. Each of these neuron populations can be distinguished by co-expression of calR±tk (Qu et al., 2008). Presumably both the reflex activity as well as the migrating motor complex would be adversely affected in the *Hand2* mutant mice (Bornstein et al., 2006). The increased population of cholinergic neurons does not appear sufficient to compensate physiologically for the overall loss of excitatory neurons. The excitatory longitudinal muscle motoneurons and ascending interneurons would be the most severely impacted as both ACh and calR are reduced. The lack of impact on expression of calB by deletion of *Hand2* in the NEP cells is very interesting because fibers positive for calB are not found in muscle but appear mainly in the mucosa in intrinsic sensory neurons (Qu et al., 2008; Hao and Young, 2009).

There was a significant increase in the number of NNEP-derived neurons expressing calB, raising the intriguing possibility that the two neural precursor populations do not share equivalent potential for neurotransmitter/neuromodulator specification. Because of the severity of the patterning defect in the mutant ENS neural network, it is difficult to determine whether neurons derived from the NNEP pool segregate to particular ganglia or plexi. It is notable that there were few cells expressing YFP in the submucosal plexus in either wild-type or *Hand2* mutant mice. This could indicate that the timing of neuron birth could affect whether or not they will localize finally to the submucosal plexus. Alternatively, it is possible that submucosal neurons are selected early and are exclusively derived from the NNEP pool in the mouse.

In conclusion, we show that the bHLH DNA-binding protein Hand2 has functional roles in both neurogenesis and acquisition of cell type-specific phenotypic characteristics in the developing murine ENS. The identification of two neural precursor cells derived from the neural crest that contribute differentially to generation of neurons and glial cells in the ENS adds some clarity to the intersection of migration and differentiation potential within the neural crest. The dramatic impact of decreased cell number and alterations in neurotransmitter specification on patterning of the myenteric neural network may provide a powerful model for future studies to identify *Hand2*-interacting genes that are functional within the developing ENS.

Acknowledgements
The authors thank Dr Phyllis Pugh for assistance with Image J and Dr Amanda Barlow for insightful comments on the manuscript. The technical assistance of Mr Sean Ehinger is also gratefully acknowledged.

Funding
Support for this work was provided by grants from the National Institutes of Health [NS040644 and NIDDK067064 to M.J.H.]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

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