

Lack of $\beta 1$ integrins in enteric neural crest cells leads to a Hirschsprung-like phenotype

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The enteric nervous system arises mainly from vagal and sacral neural crest cells that colonise the gut between 9.5 and 14 days of development in mice. Using the Cre-LoxP system, we removed $\beta 1$ integrins in the neural crest cells when they emerge from the neural tube. $\beta 1$ -null enteric neural crest cells fail to colonise the gut completely, leading to an aganglionosis of the descending colon, which resembles the human Hirschsprung's disease. Moreover, $\beta 1$ -null enteric neural crest cells form abnormal aggregates in the gut wall, leading to a severe alteration of the ganglia network organisation. Organotypic cultures of gut explants reveal that $\beta 1$ -null enteric neural crest cells show impaired adhesion on extracellular matrix and enhanced intercellular adhesion properties. They display migration defects in collagen gels and gut tissue environments. We also provide evidence that $\beta 1$ integrins are required for the villi innervation in the small intestine. Our findings highlight the crucial roles played by $\beta 1$ integrins at various steps of enteric nervous system development.

KEY WORDS: Enteric nervous system, $\beta 1$ integrins, Neural crest, Migration, Conditional knockout, Hirschsprung's disease, Mouse

INTRODUCTION

The enteric nervous system (ENS) is the part of the autonomic nervous system that regulates peristalsis, secretions, blood supply and immune response in the intestinal tract (Newgreen and Young, 2002a; Newgreen and Young, 2002b). In mice, as in humans, the ENS is composed of interconnected ganglia organised in two concentric plexuses in the gut wall: the myenteric and the submucosal plexuses. Each ganglion contains glial cells and several subtypes of neurons. In mice, the ENS arises from vagal and sacral neural crest cells (NCCs). Vagal NCCs invade the foregut at E9.5, then progress through the gut mesenchyme in a rostrocaudal direction and reach the rectum at E14 (Young et al., 1998). Sacral NCCs give rise to a fraction of neurons and glial cells in the distal intestine by colonising the hindgut from the pelvic plexus in a caudorostral wave (Kapur, 2000; Druckenbrod and Epstein, 2005).

During their progression in the gut wall and the later development of the ENS, the enteric NCCs (ENCCs, including vagal and sacral NCCs within the gut) interact with their environment, which is composed of mesenchymal cells and extracellular matrix (ECM) components, like fibronectin, laminin, tenascin and chondroitin sulfate proteoglycan (Newgreen and Hartley, 1995; Simon-Assmann et al., 1995; Rauch and Schafer, 2003). ENS precursors express various receptors for ECM, such as $\alpha 4$ (Bixby et al., 2002; Kruger et al., 2002), $\alpha 6$ and $\beta 1$ integrins (Iwashita et al., 2003) (current study), and the 110 kDa laminin receptor (Chalazonitis et al., 1997). Integrins comprise a large family of 24 $\alpha\beta$ heterodimers that can activate signalling pathways that control cell proliferation, survival, migration or

differentiation (Hynes, 2002). The $\beta 1$ integrins represent the largest subfamily, as the $\beta 1$ chain can associate with 12 different α subunits.

Although naturally occurring and targeted mutations in rodents have identified several genes implicated in ENS formation (reviewed by Newgreen and Young, 2002a; Newgreen and Young, 2002b), little is known about the role of ECM components and their receptors in the process. In mice, the homozygous disruption of the $\beta 1$ integrin gene results in an early death of the embryo at E5.5 (Fässler and Meyer, 1995; Stephens et al., 1995). The analysis of chimaeric embryos did not provide any information about the roles of $\beta 1$ integrins during ENS development (Fässler and Meyer, 1995). In order to circumvent the early lethality caused by the knockout of the $\beta 1$ integrin gene, we restricted its inactivation to the migrating NCCs, through the use of the Ht-PA-Cre mouse line. We have previously shown that this line drives the Cre-mediated recombination specifically in NCCs as they emerge from the neural tube. This allows a precocious targeting of virtually all the NCC derivatives, including the ENS (Pietri et al., 2003; Pietri et al., 2004). We show that the loss of the $\beta 1$ integrins in ENCCs causes an incomplete colonisation of the gut and an abnormal organisation of the enteric ganglia network. This phenotype is mostly due to a migration defect, linked to an increased aggregation of $\beta 1$ -null ENCCs.

MATERIALS AND METHODS

Mouse maintenance and genotyping

Crossing, maintenance and genotyping of mice were carried out as described by Pietri et al. (Pietri et al., 2004).

Samples processing and immunostaining

Immunostaining was performed as described by Pietri et al. (Pietri et al., 2004) for whole tissues and paraffin sections, and by Delannet et al. (Delannet et al., 1994) for frozen sections. The primary antibodies we used are listed in Table S1 in the supplementary material.

The β -galactosidase (β -gal) activity was detected by X-Gal staining on whole tissues as described in Dufour et al. (Dufour et al., 1994). The β -gal activity of the protein extracts was measured using the β -galactosidase Enzyme Assay System (Promega).

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For 5-bromo-2'-deoxyuridine (BrdU) incorporation, mice were injected intraperitoneally with 100 μg of BrdU/g of body weight 2 hours prior to sacrifice.

Organotypic cultures

For graft experiments, segments of control or mutant distal midgut were grafted onto segments of wild-type distal hindgut. The explants were placed on the filter of a Millicell chamber (Millipore) and cultured in growth factor-reduced Matrigel (Becton Dickinson), in DMEM/F12 medium (Invitrogen) supplemented with 5% horse serum (Babco). In orienting the explants, special care was taken to respect the rostrocaudal direction of ENCC migration. Only explants without a gap between the two grafted segments were further analysed (five independent experiments, control: $n=11$, mutant: $n=10$). The quantification of the migration was achieved by measuring the distance between the most caudal $\beta\text{-gal}^+$ cell in the wild-type hindgut and the boundary separating the two grafted segments.

For cultures on a 2D substrate, rings (500 μm thick) of distal midgut were plated on a mixture of ECM Gel (Sigma) at 150 $\mu\text{g}/\text{ml}$ and fibronectin (Sigma) at 10 $\mu\text{g}/\text{ml}$, and cultured in DMEM/F12 medium supplemented with 3% horse serum (4 independent experiments, control: $n=17$, mutant: $n=20$). For the dissociation experiments, mutant explants were incubated at 37°C either in 0.001% trypsin, 1 mM EDTA in HCMF (low trypsin-EDTA), or in 0.01% trypsin in HMF (trypsin- Ca^{2+}) (Nakagawa and Takeichi, 1995).

For cultures in collagen gel, segments of proximal midguts were placed in 1.5 mg/ml collagen type I gel (Sigma) on the filter of a Millicell chamber, and cultured in DMEM/F12 medium supplemented with Insulin-Transferrin-Selenium (Gibco), either in the presence or absence of GDNF at 10 ng/ml (Promega) (three independent experiments; control: $n=9$; mutant, $n=7$).

Semi quantitative RT-PCR and western blotting

For RT-PCR, total RNA was isolated from E13.5 guts with RNAeasy Protect Mini kit (Qiagen). RNA (1.5 μg) was reverse transcribed with Mo-MuLV reverse transcriptase (200 U, Promega) primed with random hexamers (1 μg , Roche). cDNA was subjected to PCR using murine gene-specific primers listed in Table 2 in the supplementary material. Expression levels of intercellular adhesion molecules were determined relative to $\beta\text{-gal}$ expression.

For western blotting, guts were homogenised for 45 minutes on ice in Triton X-100 1%, sodium desoxycholate 10%, SDS 0.1% in PBS with Ca^{2+} and Mg^{2+} , complemented with protease inhibitor cocktail (Roche). The extracts were then processed as described by Dufour et al. (Dufour et al., 1999). Expression levels were normalised with respect to the $\beta\text{-gal}$ activity of the extracts.

RESULTS

Targeting the ENCCs

We crossed Ht-PA-Cre; $\beta 1^+/ \beta 1^-$ and $\beta 1^{\text{fl}}/ \beta 1^{\text{fl}}$ (Potocnik et al., 2000) mice to target the $\beta 1$ gene disruption in the ENS precursors. Two genotypes were obtained: Ht-PA-Cre; $\beta 1^+/ \beta 1^{\text{fl}}$ mice, used as controls, and Ht-PA-Cre; $\beta 1^-/ \beta 1^{\text{fl}}$ mice, referred to as mutants, which show several defects of the peripheral nervous system and die around 3 weeks after birth (Pietri et al., 2004). In both control and mutant mice, $\beta\text{-gal}$ gene expression is controlled by the $\beta 1$ integrin promoter after the Cre-mediated recombination at the $\beta 1$ floxed locus. This allowed us to follow the targeted cells during embryonic and postnatal development.

In embryonic control guts, the Cre-mediated recombination targeted a population of cells that colonised the gut in a rostrocaudal wave from stage E9.5 to E14 (see Fig. S1 in the supplementary material), with similar migration timetable and radial location as the vagal NCCs (Kapur et al., 1992; Young et al., 1998; Young et al., 2004). No $\beta\text{-gal}^+$ cells were seen in the hindgut before these cells arrived at E14. The pelvic plexus was labelled similarly to that found in the guts of Ht-PA-Cre; R26R mice (Pietri et al., 2003), suggesting that sacral NCCs contributing to the hindgut ENS are also targeted

(not shown). The $\beta\text{-gal}^+$ cells expressed two markers for vagal NCCs, Phox2b and p75^{NTR} (Young et al., 1998) (not shown), and colocalised with the neuronal markers HuD and NF160 (Fig. 5) and the glial marker B-FABP (not shown) at different stages of development. Taken together, these results show that the Ht-PA-targeted enteric cells correspond to the enteric vagal and sacral NCCs (referred to as ENCCs) and to their neuronal and glial derivatives.

ENCCs aggregated into myenteric ganglia between E12.5 and E16.5 (compare Fig. S1E-H with Fig. S1C,D in the supplementary material). At E16.5, a few isolated $\beta\text{-gal}^+$ cells were found in the submucosal zone of the small intestine, which are likely to belong to the future submucosal plexus (Fig. S1F). However, in the colon, no labelled cells were observed in this zone (Fig. S1H). This confirms that the submucosal plexus appears earlier in the small intestine than in the colon, as described before (McKeown et al., 2001). In E16.5 small intestines, $\beta\text{-gal}^+$ cells were observed, the nuclei of which appeared to be passing through the circular muscle layer. It is likely that they were migrating from myenteric ganglia towards the submucosal zone (Fig. S1I).

The $\beta 1$ -null ENCCs fail to colonise the entire gut

In conditional mutants, $\beta 1$ integrins were barely detectable at the cell surface of mutant ENCCs when they start to invade the foregut, at E9.5 (Fig. 1A-D), and completely absent from E11.5 (Fig. 1E-H). Thus, our genetic system permits the efficient removal of the $\beta 1$ integrins from the beginning of the gut colonisation by ENCCs. The $\beta 1$ integrin loss did not have a strong influence on the expression of other β integrins, as $\beta 3$ and $\beta 5$ integrins were similarly expressed in mutant and control ENCCs (Fig. 1I-P).

In all the mutant animals (number of X-Gal-stained samples: mutant, $n=37$; control, $n=48$), the ENCCs showed a delay in the gut colonisation, detected from E11.5. At this stage, the control ENCCs migratory front was located at the base of the caecum, although the mutant front was still located in the distal midgut (not shown). At E12.5, chains of control ENCCs had invaded the hindgut, whereas mutant ENCCs had entered the proximal caecum (Fig. 2A,B). At E16.5, control ENCCs had reached the rectum, whereas the $\beta 1$ -null ENCC migratory front was located in the middle of the hindgut (Fig. 2C,D). Thus, the difference in the distance travelled by control and mutant ENCCs increased with time. This defect did not appear to be just a delay, as the $\beta 1$ -null ENCCs did not subsequently invade the caudal hindgut, leading to an aganglionosis of the descending colon after birth that resembles the human Hirschsprung's disease (HSCR) (Passarge, 2002) (Fig. 2E,F). The majority of the new-born mutants had a distended ascending colon and caecum (megacolon) (Fig. 2G,H). In addition, as it has been documented in HSCR, bundles of extrinsic neurons innervated the aganglionic segments (Fig. 2F). These bundles were $\beta\text{-gal}^+$ and expressed tyrosine hydroxylase (not shown), revealing their sympathetic neuronal origin. We did not detect any anomaly in the radial location of ENCCs during their progression in the gut, or any delay in the formation of the submucosal plexus in the mutant (not shown). Thus, in contrast to the migration across the longitudinal axis, the loss of $\beta 1$ integrins does not affect the radial distribution of ENCCs in the gut.

The $\beta 1$ -null ENCCs show increased aggregation properties

In addition to their inability to completely colonise the intestinal tract, the mutant ENCCs gave rise to an abnormal ganglia network. At E12.5, mutant ENCCs began to form clusters of cells in the distal

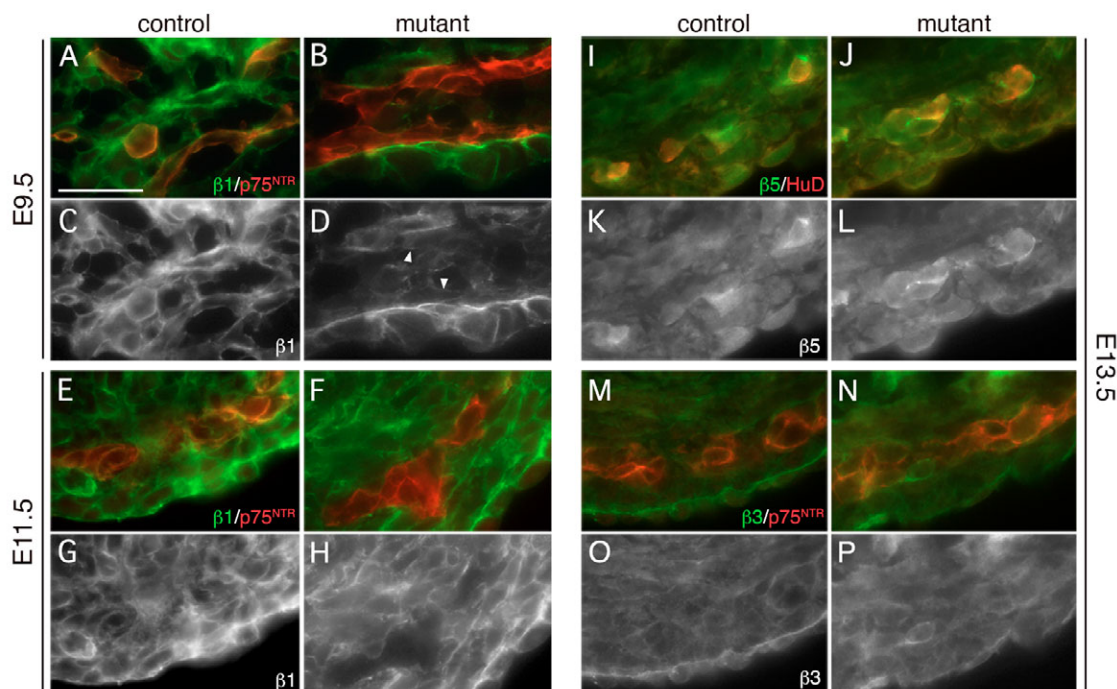


Fig. 1. Loss of β1 integrin in mutant ENCCs and expression of other β integrins. ENCCs, or neurons and their precursors, are detected with p75^{NTR} and HuD, respectively. (A-H) Frozen sections of control and mutant guts stained with anti-β1 integrin (green) and anti-p75^{NTR} (red) antibodies. (A-D) At E9.5, the β1 integrin loss is mostly complete in mutant p75^{NTR}+ cells, even though a few of them still express small amounts of β1 at their surface (D, arrowheads). (E-H) At E11.5, mutant p75^{NTR}+ cells have completely lost the β1 integrin subunit. (I-P) Frozen sections of E13.5 control and mutant midguts stained for β5 integrin (green) and HuD (red) (I-L), or for β3 integrin (green) and p75^{NTR} (red) (M-P). Control and mutant ENCCs express high levels of β5 integrin and small amounts of β3 integrin. Scale bar: 50 μm.

midgut and the caecum (Fig. 2A,B). At E16.5 and thereafter, this defect was seen in the different parts of the mutant guts, with ENCCs forming abnormal aggregates surrounded by ENCC-free spaces (Fig. 2K,M,O, compare with J,L,N). As a consequence, the organisation of the enteric network was severely altered at P14 (Fig. 2P,Q).

This increased ENCC aggregation could arise from a modification of the balance between the cell matrix and intercellular adhesion systems in β1-null ENCCs. Intercellular adhesion is mainly mediated by two systems, CAM-Ig and cadherins (cad), which are Ca²⁺ independent and Ca²⁺ dependent, respectively. We therefore analysed the in vivo expression of type-I cadherins (E-cad, N-cad), type-II cadherins (Cad6 and Cad11) and members of the CAM-Ig superfamily (NCAM and L1-CAM) in control and mutant guts, when ENCCs aggregate into ganglia. E-cad expression was restricted to the epithelium (not shown). At E15.5, myenteric ganglia expressed N-cad, Cad6, Cad11, NCAM and L1-CAM in both types of guts (Fig. 3B,D,G,I,K). At E11.5, isolated ENCCs located near the migratory front were already expressing all these proteins except L1-CAM (Fig. 3A,C,F,H,J). Analysis of immunofluorescence labelling by a deconvolution software at E13.5 further suggested a cell surface localisation for N-cad, in contrast to Cad6, which was mainly found in intracellular structures, in both types of guts (Fig. 3E). Thus, no significant differences were detected in the expression pattern or subcellular localisation of these adhesion molecules between mutant and control ENCCs, by immunofluorescence. In addition, western blot for N-cad and semi-quantitative RT-PCR for all transcripts encoding adhesion molecules did not reveal any significant modification of their expression level in mutant ENCCs (not shown). However, in dissociation experiments, the cohesion of

the in vitro mutant spheroid aggregates (see Fig. 7M,N) was found to be mediated by Ca²⁺-dependent mechanisms, suggesting that cadherins are implicated in their formation (see Fig. S2 in the supplementary material). Thus, the perturbation of intercellular adhesion properties in β1-null ENCCs is not apparently due to a change in the prevalence of intercellular adhesion proteins at the surface. However, the functional perturbation of Ca²⁺-dependent mechanisms suggests a role for cadherins in the ENCC-aggregation phenotype.

The defects of the mutant ENS are not due to a decreased number of migrating ENCCs

The incomplete gut colonisation by mutant ENCCs could be explained by a decrease in their number during migration, owing to survival and/or proliferation defects. However, apoptotic neurons were not detected either in control or mutant guts at E13.5 (Fig. 4A,B) and other stages (E10.5 and E16.5, not shown). To compare the proliferation state of control and mutant ENCCs, we analysed their expression of the PCNA antigen (Fig. 4C,D) and their BrdU incorporation (Fig. 4F,G) at E12.5. No significant difference was found in the percentage of proliferative ENCCs between mutant and control with these two methods (Fig. 4E,H). Moreover, the β-gal activity measured in protein extracts prepared from E14.5 guts was equivalent between mutants (15.13±1.79 units/g protein) and controls (16.34±1.56 units/g protein), supporting the fact that the number of ENCCs is not significantly reduced in mutants. Taken together, these results strongly suggest that the partial gut colonisation by mutant ENCCs is due to a migration and/or a differentiation defect, and not to survival or proliferation alteration decreasing their number.

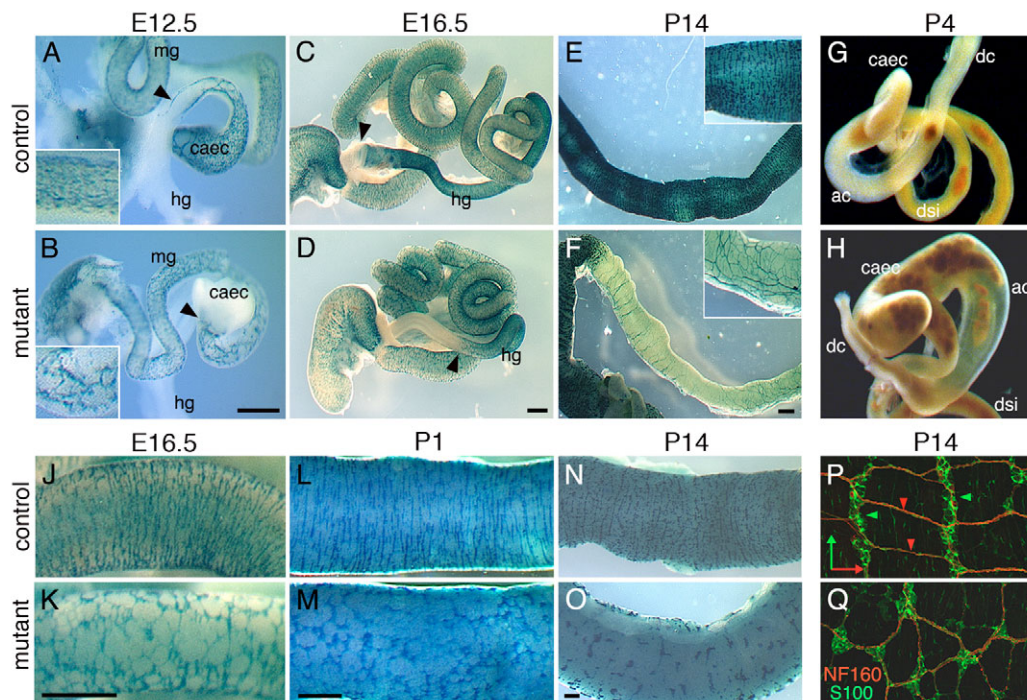


Fig. 2. ENS defects in $\beta 1$ integrin conditional mutants. (A-F) Whole-mount X-Gal staining. (A,B) E12.5 guts. Black arrowheads indicate the position of the ENCC migratory front. The control hindgut is being invaded, whereas the mutant ENCC front is still located in the proximal caecum. Insets show higher magnification of distal midguts. ENCCs have a scattered distribution in the control but form clusters in the mutant. (C,D) E16.5 guts. The mutant ENCCs have stopped in the middle of the hindgut, whereas control ENCCs have reached the rectum (black arrowheads). (E,F) Descending colon at P14, with higher magnification in insets. Aganglionosis occurs in the mutant, with abnormal extrinsic innervation, compared with the control regular network. (G,H) Freshly dissected P4 guts. The mutant ascending colon and caecum are distended (megacolon) compared with control. (J-O) Whole-mount X-Gal staining of small intestines at E16.5 (J,K), P1 (L,M) and P14 (N,O). Mutant ENCCs form abnormal aggregates surrounded by ENCC-free spaces, compared with the regular ganglia network in the control. (P,Q) Confocal images of a whole-mount immunostaining on P14 small intestines with anti-NF160 (red) and anti-S100 (green) antibodies, recognising neuronal processes and glial cells, respectively. Green and red arrows in P indicate the circular and longitudinal orientations of the samples, respectively. Green and red arrowheads show control ganglia and neuronal processes following the circular and longitudinal directions. In the mutant, ganglia and neuronal processes do not follow these directions. ac, ascending colon; caec, caecum; dc, descending colon; dsi, distal small intestine; hg, hindgut; mg, midgut. Scale bar: 500 μ m in B,D,K; 2 mm in F; 1 mm in M,O.

The mutant ENCCs differentiate normally but are strongly associated with neuronal processes

It has been suggested that the ability of ENCCs to migrate is inversely correlated with their neuronal differentiation (Wu et al., 1999; Young et al., 2004). We therefore examined the neuronal differentiation of ENCCs in relation to the gut colonisation, by labelling the neuron cell bodies and the neuronal processes on E12.5 X-Gal stained guts (Fig. 5). In both types of guts, the neuronal differentiation front, represented by the most caudal HuD^+ cell body, was located just rostral to the migratory front. Thus, the leading $\beta\text{-gal}^+$ cells were not HuD^+ , suggesting that they were in an undifferentiated state, in controls as well as in mutants (Fig. 5E,F). The proportion between neurons and differentiating neurons ($\beta\text{-gal}^+\text{HuD}^+$) and undifferentiated ENCCs ($\beta\text{-gal}^+\text{HuD}^-$) was similar in control and mutant guts, near the migratory front and elsewhere (not shown). ENCCs at the migratory front were closely associated with NF160^+ neuronal processes (Fig. 5E,F), suggesting that the progression of ENCCs in the gut is linked to neuronal processes elongation. Rostral to the front, in the distal midgut, control neurons and non-neuronal ENCCs formed a scattered network occupying all the available space (Fig. 5C). By contrast, $\beta 1$ -null neurons and other ENCCs were strongly associated with fasciculated neuronal processes, forming thick bundles surrounded by ENCC-free areas

(Fig. 5D). Moreover, the position of the glial differentiation front analysed with the B-FABP marker, was similar in both types of guts at E12.5 (not shown). Taken together, these results suggest that the timing of the neuronal and glial differentiation is not affected in mutants. Moreover, analysing the neuronal differentiation revealed an abnormal association between mutant ENCCs and neuronal processes. This may reflect their incapacity to explore and occupy the gut environment properly.

We then examined the acquisition of different neuronal subtypes (VIP, NPY, SP, NOS and CGRP) by control and mutant neurons. At P7, all these markers were expressed in both control and mutant enteric ganglia (not shown), showing that $\beta 1$ -null neurons retain the capacity to differentiate in various neuronal subtypes. However, at P7 and P4, the VIP staining revealed an innervation defect of the villi in the mutant small intestines, probably resulting from a neuronal process degeneration (see Fig. S3 in the supplementary material).

The $\beta 1$ -null ENCCs show a migration defect

Our results concerning ENCC apoptosis, proliferation and differentiation strongly suggest a migration defect. In order to compare the migratory behaviours of control and mutant ENCCs in a 3D tissue environment, we performed graft experiments at E12.5. Segments of distal midgut, already colonised by ENCCs, were

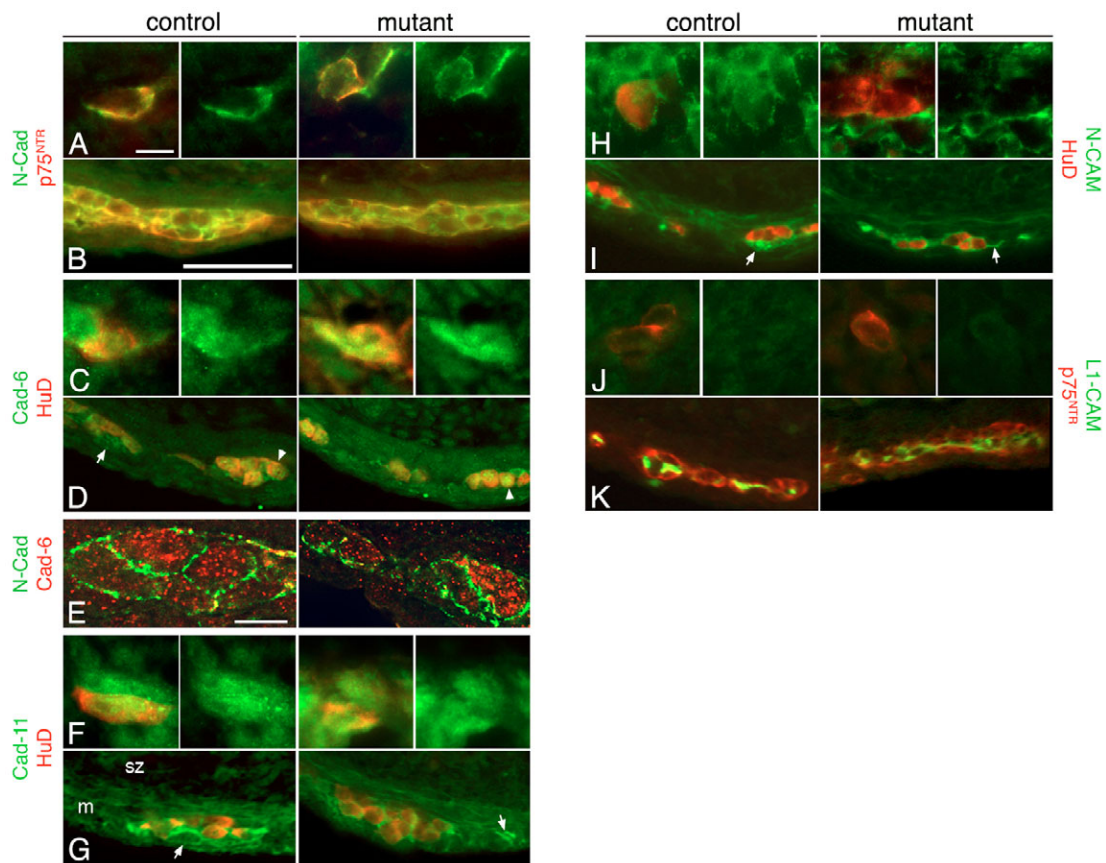


Fig. 3. Expression pattern of intercellular adhesion proteins in ENCCs. Immunolocalisation of intercellular adhesion proteins (in green) on frozen sections of E11.5 distal midgut (A, C, F, H, J) and paraffin sections of E15.5 small intestine (B, D, G, I, K). ENCCs, or neurons (in green), are detected with p75^{NTR} (in red, A, B, J, K) and HuD (in red, C, D, F-I), respectively. (B) N-cad is expressed at the same level by all the neurons and glial cells of the E15.5 myenteric ganglia (p75^{NTR+} cells). (D) At E15.5, Cad6 is expressed at high levels by a population of myenteric neurons (HuD⁺ cells, white arrowheads), and at lower levels by other neurons and HuD⁻ cells, closely associated to neurons, very likely to be glial cells (white arrow). (F-I) Cad11 and NCAM are expressed by both neurons and glial cells (white arrows), and at lower level by the smooth muscles and the submucosal zone. (K) L1-CAM is detected in fibre-like structures in the E15.5 p75^{NTR+} myenteric ganglia, likely to be neuronal processes. All these proteins, except L1-CAM, are already expressed by isolated ENCCs located near the migratory front at E11.5 (lines A, C, F, H, J). (E) Deconvolution analysis shows a cell surface localisation for N-cad (in green) and intracytoplasmic accumulation of Cad6 (in red) in E13.5 ENCCs. m, smooth muscles; sz, submucosal zone. Scale bar: 5 μm for A, C, E, F, H, J; 50 μm for B, D, G, I, K.

grafted onto segments of wild-type distal hindgut, devoid of ENCCs (Fig. 6A). After 3 days in culture, the explants were stained with X-Gal to label the ENCCs that had entered the wild-type hindguts. Both control and mutant ENCCs were able to enter the wild-type hindguts, but the distance travelled by the β1-null ENCCs was significantly reduced (Fig. 6B-E). On average, mutant ENCCs covered 66.5% of the control ENCC distance, showing that β1-null ENCCs lost part, but not all, of their migratory abilities, as suggested by the in vivo mutant phenotype. Moreover, these data indicate that the distal hindgut environment is not repellant to β1-null ENCCs.

To compare the extent of adhesion and migration of the mutant and control ENCCs on a 2D substrate, rings of E13.5 distal midgut were plated on a matrix that mimicks the gut ECM. In control cultures, neurons and glial cells formed scattered neuronal networks around the explants (Fig. 7A), either on the smooth muscle cells (SMC) (Fig. 7B,C) or directly on ECM (Fig. 7D-G). By contrast, only few mutant ENCCs were found outside the explants (Fig. 7H), and their most frequent behaviour was to form aggregates containing both neurons and glial cells and linked to the explants by neuronal processes. Few of them were adhering to the SMC layer (Fig. 7I,J),

or to the ECM substrate (Fig. 7K,L), but the majority floated in the medium (Fig. 7H), with a spheroid shape (Fig. 7M,N). Thus, instead of forming scattered neuronal networks, the mutant ENCCs formed aggregates. This revealed an impaired ability of the β1-null ENCCs to interact efficiently with their environment.

To further characterise this migration defect, we cultured segments of E13.5 proximal midguts in 3D collagen gels, in the presence or absence of GDNF, a known chemoattractant for ENCCs. In the absence of GDNF, no cell was found outside the explants, in both control and mutant cultures (Fig. 8A,B,E,F). As previously described (Natarajan et al., 2002; Iwashita et al., 2003), in the presence of GDNF, a large number of control neurons (both cell bodies and processes) and glial cells emigrated out of the explant (Fig. 8C,D). By contrast, in the mutant, neuronal processes had penetrated into the collagen gel, but not neuron cell bodies or glial cells (Fig. 8G,H). These results confirm our in vivo and in vitro data about the alteration of the β1-null ENCC migratory abilities. In addition, they support the idea that neurite outgrowth of enteric neurons is β1 integrin independent, suggesting that ENCC migration and neurite extension in response to GDNF are regulated by distinct mechanisms.

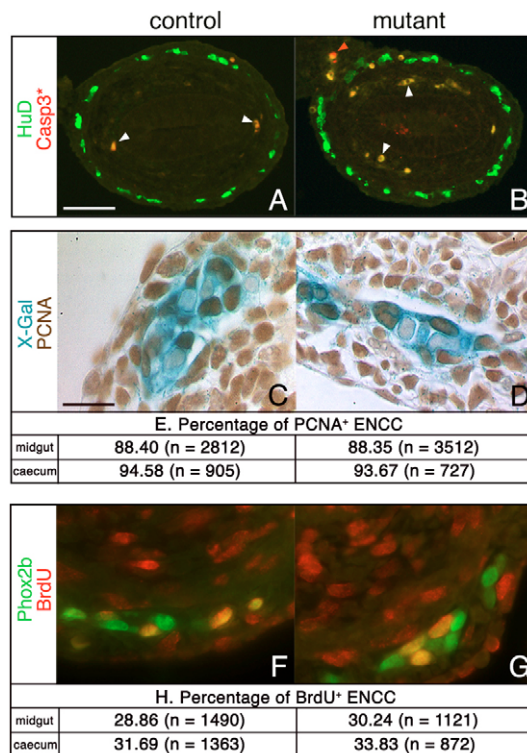


Fig. 4. Apoptosis and proliferation of ENCCs. (A,B) Activated caspase 3 (red) and HuD (green) double immunostaining on E13.5 midgut sections. No apoptotic neuron is detected in mutant and control. White arrowheads indicate autofluorescent red blood cells in the submucosal zone. The red arrowhead shows an activated-caspase-3⁺ HuD⁻ cell in the mesentery. (C,D) PCNA immunostaining on E12.5 X-Gal-stained gut sections. (F,G) BrdU (red) and Phox2b (green) double immunostaining on E12.5 gut sections. (E,H) Percentages of β -gal⁺ cells that were PCNA⁺ (E) and percentages of Phox2b⁺ cells that were BrdU⁺ (H), in control and mutant midgut and caecum (cell counts from several preparations were pooled; n, total number of ENCCs examined; χ^2 test, $P > 0.05$). Scale bar: 100 μ m in A,B; 20 μ m in C,D,F,G.

DISCUSSION

β 1 Integrins do not control the number of migrating ENCCs

Anchorage dependence has long been recognised as a condition for cell survival, as integrins activate the pro-survival PI3 kinase and MAP kinase pathways (Stupack and Cheresch, 2002). Neuron survival depends on β 1-mediated cell-matrix contacts in vitro (Rozzo et al., 1997; Bonfoco et al., 2000; Gary et al., 2003). In addition, α 4 β 1 and α 5 β 1 integrin are required for in vitro peripheral glial cell survival and proliferation, respectively (Haack and Hynes, 2001). We have shown that the loss of the β 1 integrins in ENCCs does not affect their survival and proliferation during the gut colonisation. Moreover, because our data suggest that the number of ENCCs is not decreased in mutants, a reduction in the pool of vagal NCCs before their entry in the gut is unlikely to contribute to the phenotype. Indeed, the complex environment in vivo probably counteracts the apoptotic effect due to the loss of β 1 integrins in vitro. β 1-Null ENCCs may receive signals through growth factor receptors, other ECM receptors and/or intercellular adhesion molecules that are sufficient for their survival and proliferation. In particular, both control and mutant ENCCs were found to express the β 5 integrin chain, and small amounts of β 3 integrin, at E11.5

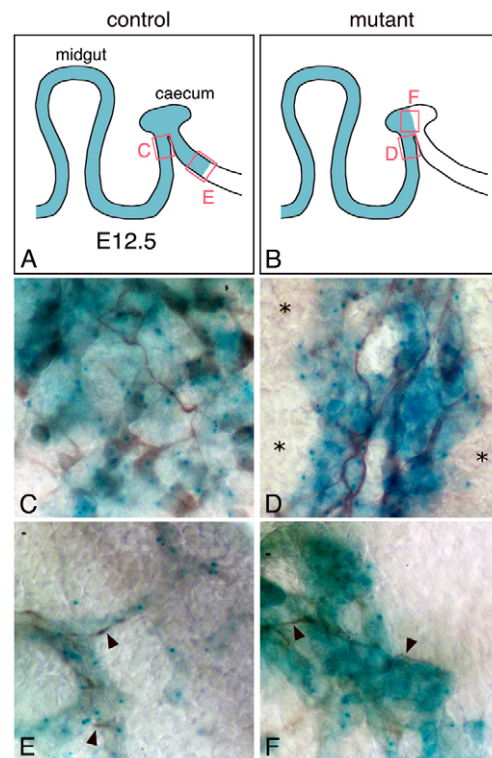


Fig. 5. Neuronal differentiation of ENCCs. (A,B) Schematic representation of the E12.5 X-Gal stained guts which were immunostained with anti-NF160 and anti-HuD antibodies, recognising neuronal processes and neuron cell bodies, respectively. The regions which are colonised by ENCCs are stained in blue. The red boxes indicate the regions where pictures C-F were taken. (C,D) Distal midgut. (C) Control neurons and non-labelled β -gal⁺ ENCCs form a scattered network occupying all the available space. (D) Mutant neurons and non-labelled β -gal⁺ ENCCs form bundles around fasciculated neuronal processes, surrounded by ENCC-free areas indicated by asterisks. (E,F) Migratory front. Black arrowheads indicate NF160⁺ processes associated with the leading ENCCs, in control and mutant.

(not shown) and E13.5 (Fig. 11-P). Moreover, N-cadherin, which is expressed by β 1-null ENCCs, is known to activate pro-survival signalling pathways (Li et al., 2001; Tran et al., 2002).

β 1 Integrins are required for the migration of ENCCs along the gut

The mutant ENCCs show a time-increasing delay in the gut colonisation from E11.5, leading to an aganglionosis restricted to the descending colon. The β 1-null ENCCs do not migrate efficiently in vitro and ex vivo, strongly suggesting that this incomplete gut colonisation is due mostly to a migration defect (both persistence and speed of locomotion appear to be altered in mutant ENCCs) (M.A.B., unpublished). This reveals the crucial role of β 1 integrins in the migration of this NCC population in vivo.

The spatial restriction of the aganglionosis to the descending colon is not due to the late requirement of β 1 integrins at the time of distal hindgut invasion, because (1) our graft experiments suggest that the distal hindgut environment is permissive for β 1-null ENCC migration, and (2) the delay of the mutant ENCCs is observed from E11.5, well before the hindgut colonisation. This delay increases with time. This could be due to a progressive loss of the β 1 integrin subunit at the ENCC surface. However, it is unlikely, because the β 1

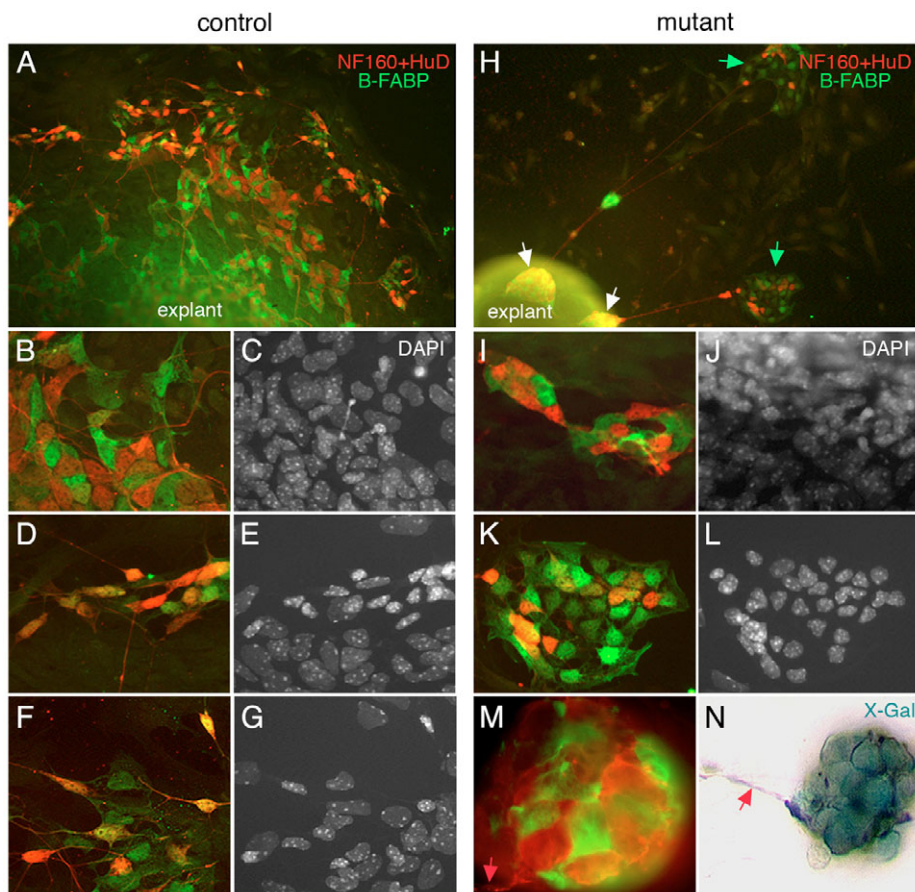


Fig. 7. The mutant ENCCs form aggregates on a 2D substrate. Rings of E13.5 control and mutant distal midgut were plated on a mixture of ECM gel and fibronectin. After 2 days in culture, neuron cell bodies were detected with HuD (red), neuronal processes with NF160 (red), glial cells with B-FABP (green) and nuclei with DAPI. **(A-G)** Control culture, at low (A) and high (B-G) magnification. Neurons and glial cells form scattered networks on SMC (B,C) or directly on ECM (D-G). **(H-N)** Mutant culture, at low (H) and high (I-N) magnification. Green arrows indicate aggregates of neurons and glial cells that adhere directly to ECM. White arrows show spheroid aggregates that do not adhere to SMC or ECM. (I,J) Aggregates on the SMC layer. (K,L) An aggregate adhering to ECM. (M,N) High magnifications of spheroid aggregates stained for HuD and NF160 (red) and B-FABP (green) (M) and for X-Gal (N). Red arrows indicate neuronal processes linking aggregates to the explants.

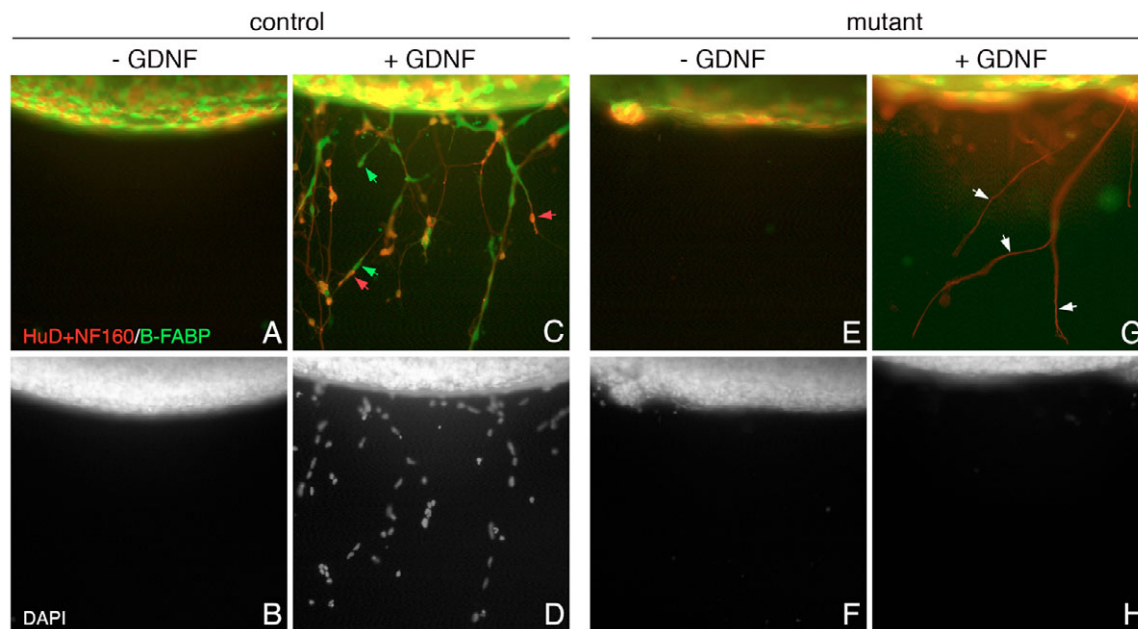


Fig. 8. The ENCC migratory response to GDNF is $\beta 1$ integrin dependent. Segments of E13.5 proximal midgut were embedded in 3D collagen matrix and cultured in the presence or absence of GDNF. After 2 days of culture, neuron cell bodies were detected with HuD (red), neuronal processes with NF160 (red), glial cells with B-FABP (green) and nuclei with DAPI. **(A,B,E,F)** Without GDNF, no ENCCs migrated out of the explants. **(C,D)** In the presence of GDNF, a large number of neurons (red arrows) and glial cells (green arrows) emigrated from control explants. Neurons and glial cells were the only cell types found out of the explants, as shown by the DAPI staining. **(G,H)** In the mutant, no neuron cell bodies or glial cell entered the collagen matrix in the presence of GDNF, but neuronal processes did (white arrows).

layers. In E16.5 control small intestines, we saw cells belonging to the myenteric plexus migrating towards the submucosal zone. This is consistent with the hypothesis of a radial migration of myenteric ENCCs towards the epithelium, giving rise to the submucosal plexus (Burns and Le Douarin, 2001; McKeown et al., 2001). As no anomaly in the submucosal plexus formation was detected in mutants, it seems that β1 integrins are not required for this radial ENCC migration. However, β1 integrins are necessary for the innervation of the small intestine epithelial villi after birth. Nevertheless, the neuronal processes innervated the full length of the villi at earlier stages (see Fig. S3E-H in the supplementary material), suggesting that this alteration is not due to a neurite outgrowth defect during development but rather to a degeneration of neuronal processes.

β1 Integrins and intercellular adhesion in ENCCs

ENCCs lacking β1 integrins do not lose all their migratory abilities, suggesting a role for other ECM receptors, such as the 110 kDa laminin receptor (Chalazonitis et al., 1997) and β3 and β5 integrins, or intercellular adhesion molecules in their migration through gut tissues. The importance of intercellular adhesion in ENCC progression is supported by the various repertoire of intercellular adhesion proteins we found to be expressed by ENCCs. In addition, a recent time-lapse microscopy study of the migration revealed the importance of intercellular contacts between migrating and post-migrating ENCCs (Young et al., 2004). In the case of ENCCs, intercellular adhesion and migration must not be antagonistic processes. Their chain migration may depend on a delicate balance between cell-matrix and intercellular adhesion. The increased aggregation of mutant ENCCs correlated to their impaired adhesion to ECM suggest that this balance is perturbed in β1-null ENCCs. Several studies have described crosstalk between β1 integrins and intercellular adhesion systems, such as cadherins, *in vitro* (Monier-Gavelle and Duband, 1997; Yano et al., 2004; Schaller, 2004) and *in vivo* (Marsden and De Simone, 2003). However, we have found no difference in the pattern and level of expression of the intercellular adhesion proteins between control and mutant ENCCs. Nevertheless, dissociation experiments of mutant aggregates suggested that cadherins are implicated in their formation. Further analysis is required to elucidate the mechanisms by which cadherins enhance intercellular interactions in β1-null ENCCs, such as mobilisation of a cytoplasmic pool or activation of cadherins at the cell surface.

As it is known that strong cell-cell contacts can inhibit cell migration (Monier-Gavelle and Duband, 1997; Dufour et al., 1999), the abnormal aggregation of β1-null ENCCs could participate to their migration defect. Moreover, it has been suggested that a high ENCC density promotes their migration (Young et al., 2004). The diminution of the population pressure caused by the mutant ENCC aggregation could also be an additive mechanism by which they do not reach the caudal hindgut.

β1 Integrins and HSCR

HSCR is the most common human congenital defect affecting the ENS (1 in over 5000 births). It consists of a distal aganglionosis of variable length, leading to a potentially fatal intestinal obstruction (Passarge, 2002). The mutations responsible for half of the cases have not been identified yet, and to date, no mutation of the β1 integrin gene has been reported in individuals with HSCR. As the β1 integrin is ubiquitously expressed, complete loss-of-function mutations of the gene must be lethal at early stages of human development, as in the mouse. Two signalling systems, mediated by

the receptor tyrosine kinase Ret and the G-protein-coupled receptor Ednrb, have been shown to play crucial roles in ENS development (Hearn et al., 1998; Barlow et al., 2003; Kruger et al., 2003). Integrin signalling could interact with the signalling pathway of these receptors. In particular, the endothelin pathway is known to interact with cell matrix and intercellular adhesion systems (Koyama et al., 2003; Bagnato et al., 2004). Moreover, mutations in genes coding for endothelin 3 or its receptor Ednrb give rise to defects which are similar to the β1 mutant phenotype (Kapur et al., 1992; Wu et al., 1999). Taken together, these observations suggest a possible interaction between β1 integrin and endothelin 3 signalling pathways in ENCCs.

Our study demonstrates that the removal of β1 integrins in ENCCs leads to a partial colonisation of the intestinal tract by ENCCs and a severe alteration of the ENS network organisation. These defects are mostly due to a defective migration linked to an increased cell aggregation. Our study also reveals a role for β1 integrins in the late maintenance of villi innervation. In conclusion, β1 integrins are crucial at various key steps of the ENS development.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/9/1725/DC1>

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