Cell Players in Gut Motility Review Series *J. Cell. Mol. Med. Vol 13, No 7, 2009 pp. 1193-1210*

Guest Editor: M. S. Faussone-Pellegrini

Development of enteric neuron diversity

Marlene M. Hao, Heather M. Young *

Department of Anatomy & Cell Biology, University of Melbourne, Parkville, Victoria, Australia

Received: April 20, 2009; Accepted: May 22, 2009

Abstract

The mature enteric nervous system (ENS) is composed of many different neuron subtypes and enteric glia, which all arise from the neural crest. How this diversity is generated from neural crest-derived cells is a central question in neurogastroenterology, as defects in these processes are likely to underlie some paediatric motility disorders. Here we review the developmental appearance (the earliest age at which expression of specific markers can be localized) and birthdates (the age at which precursors exit the cell cycle) of different enteric neuron subtypes, and their projections to some targets. We then focus on what is known about the mechanisms underlying the generation of enteric neuron diversity and axon pathfinding. Finally, we review the development of the ENS in humans and the etiologies of a number of paediatric motility disorders.

Keywords: enteric nervous system • Hirschsprung's disease • neural crest • neuronal differentiation • paediatric motility disorders

Neuronal diversity in the adult enteric nervous system (ENS)

The ENS is composed of a complex network of neurons plus an equal or higher number of enteric glial cells [1, 2]. Enteric neurons form complex circuits that regulate or control a variety of gut functions including motility, secretion, vascular tone and release of hormones. Although there is normally interplay between the ENS and the central nervous system (CNS), in most regions of the gastrointestinal tract the ENS can function autonomously; the ENS has therefore been referred to as a 'second brain' [3]. In mammals and birds, most enteric neurons are clustered in myenteric ganglia (Fig. 1A), which are located between the circular and longitudinal muscle layers, and in submucosal ganglia. In the intestine of smaller mammals, myenteric neurons are primarily involved in the

*Correspondence to: Heather M. YOUNG,
Department of Anatomy & Cell Biology,
University of Melbourne, 3010 Australia.
Tel.: 613-8344-0007
Fax: 613 9347 5219
E-mail: h.young@unimelb.edu.au

doi:10.1111/j.1582-4934.2009.00813.x

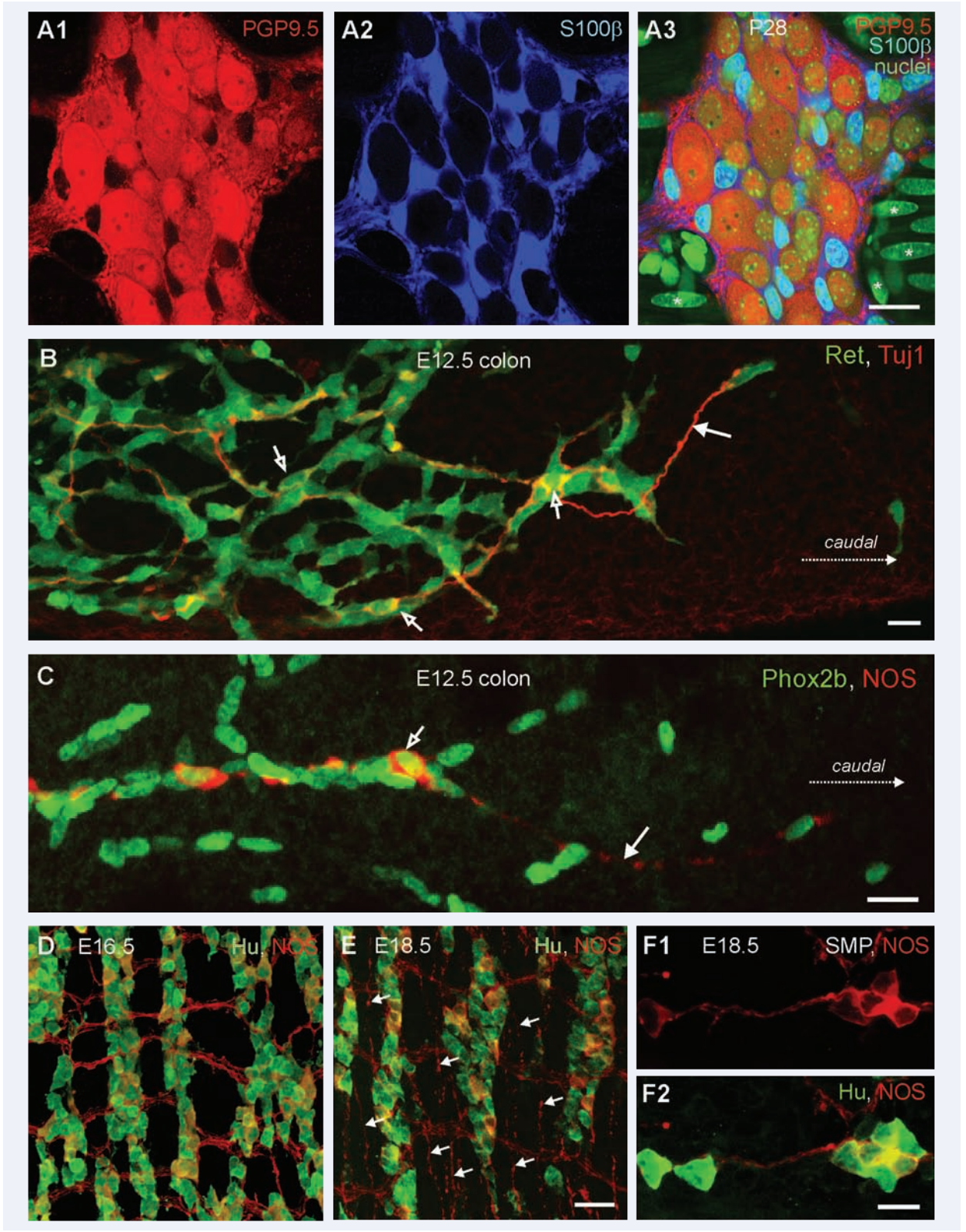

Fig. 1 (**A1–3**) Single optical section through a myenteric ganglion in wholemount preparation of colon from a 28-day-old mouse following immunostaining performed with the pan-neuronal marker, PGP9.5 (*red*) and the glial marker, S100β (*blue*). The nuclei had been stained using the nucleic acid stain, SYTO (*green*). The nuclei adjacent to the ganglion (*asterisks*) belong to fibroblasts, interstitial cells of Cajal or circular smooth muscle cells. (**B**) Wholemount preparation of colon from an E12.5 Ret^{TGM} mouse [219] in which the neural crest cells express GFP that had also been immunostained performed with the pan-neuronal marker, Tuj1. $Tuj1^{+}$ cell bodies (*open arrows*) are intermingled with other crest-derived (GFP^{+}) cells, and they project axon-like processes (*arrows*) in close association with migrating crest cells. (**C**) Wholemount preparation of colon from an E12.5 mouse following staining with the pan-neural crest cell marker, Phox2b (*green*), and NOS (*red*). Some of the NOS cell bodies (*open arrow*) give rise to axon-like processes (*arrow*) that project caudally. (**D, E**) Wholemount preparations of small intestine from E16.5 (**D**) and E18.5 (**E**) mice immunostained with the pan-neuronal marker, Hu (*green*), and the neuron subtype marker, NOS (*red*). NOS fibres (*arrows*) are present in the circular muscle of E18.5 mice (**E**), but not in E16.5 (**D**) mice. These fibres run perpendicular to the first nerve fibre tracts to form, which project longitudinally (see Fig. 2E). (**F**) Submucosal neurons in the small intestine of an E18.5 mouse immunostained for Hu (*green*) and NOS (*red*). Although only around 1% of submucosal neurons in adult mice express NOS [19], approximately 50% of submucosal neurons in late embryonic and early post-natal stages express NOS [78]. All scale bars except D, E = 25 μm; D, E = 50 μm.

regulation of gut motility and submucosal neurons are mostly involved in the regulation of secretion and vascular tone; in larger mammals, some submucosal neurons are also directly involved in motility reflexes [4].

Initially, enteric neuron subtypes were identified by Dogiel based on morphological characteristics alone (see [1]). However, elegant studies performed in the past few decades utilizing a variety of immunohistochemical, electrophysiological, pharmacological and tracing techniques have identified different subtypes of enteric neurons based on a combination of morphology, neurotransmitters, electrophysiology, target tissue and direction (up, down or circumferentially) and length of axon projection.

Enteric neurons have been most thoroughly studied in the guinea-pig ileum, where 10–15 subtypes of myenteric neurons and 4–5 subtypes of submucosal neurons have been identified; these include intrinsic primary afferent (sensory) neurons, ascending and descending interneurons, excitatory and inhibitory motor neurons to both the longitudinal and circular muscle layers, intestinofugal, secretomotor and vasodilator neurons [5–7]. More recent studies of myenteric neurons in the mouse small intestine have shown many similarities between neuron subtypes present in guinea-pigs and mice, which include (*i*) putative intrinsic primary afferent neurons have Dogiel type II morphology and contain cholinergic markers, (*ii*) excitatory circular muscle motor neurons contain cholinergic markers and tackykinins such as substance P, (*iii*) inhibitory motor neurons contain nitric oxide synthase (NOS), the synthetic enzyme for nitric oxide, as well as vasoactive intestinal peptide (VIP) [8–11], (*iv*) there are multiple subtypes of descending interneurons (four in the guinea-pig and probably three in the mouse) but only a single class of ascending interneurons [11], (*v*) separate populations of 5-hydroxytryptamine (5-HT) and somatostatin interneurons [8, 11–14] – the 5-HT neurons in both species project anally [15] and the somatostatin interneurons in the mouse are also presumed to project anally [11] as they do in the guinea-pig [16]. Pharmacological studies provide support to the view that the neurotransmitters involved in peristaltic reflexes are highly conserved between different mammalian species [17]. There are, however, some differences between subtypes of myenteric neurons in guinea-pig and mouse, particularly in the expression of calcium binding proteins. For example, in the guinea-pig, calretinin is expressed by ascending interneurons and longitudinal muscle motor neurons [5–7], whereas in the mouse, calretinin is expressed by excitatory muscle motor neurons, ascending interneurons and some intrinsic sensory neurons [8, 11]. In the guinea-pig ileum, calbindin is expressed by a single subtype of myenteric neuron that have Dogiel type II morphology and are intrinsic sensory neurons [18], whereas in the mouse, calbindin is expressed by at least two different subtypes of neurons based on morphology and co-localization with other neurochemicals [8, 11]. Five types of submucosal neurons have been identified in the mouse small intestine, but unlike guinea-pig and other species, these do not appear to include Dogiel type II neurons [19].

Studies of the ENS of the rat [20, 21], pig [22–24] and sheep [25, 26] have revealed many similarities, but also some differences from the guinea-pig and mouse ENS. For example, in the pig small intestine, the longitudinal muscle layer is innervated by both myenteric and submucosal neurons, whereas in smaller mammals only myenteric neurons innervate the longitudinal muscle [27]. Information is also rapidly accumulating about the zebrafish ENS [28, 29]. Two differences between the ENS of fish and mammals are that in fish there are no submucosal ganglia, and myenteric neurons are not clumped into ganglia [30].

Most of the neurotransmitters (*e.g.* enkephalin, VIP, substance P), neurotransmitter synthetic enzymes (*e.g.* NOS and choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine) and other molecules that are differentially expressed in different subtypes of enteric neurons (*e.g.* the calcium binding proteins, calretinin and calbindin), are present in sub-populations of neurons in the human ENS [31–40]. Nearly 50% of myenteric neurons in the human colon contain ChAT and around 40% contain NOS [41]. Although some myenteric neurons in the human have been characterized based on morphology, projections and neurochemical staining [34, 35, 42], the subtypes of neurons present in the ENS of any region of the adult human gastrointestinal tract have not yet been systematically catalogued.

The assembly of functioning neural circuits within the ENS is dependent on the generation of specific neuronal subtypes and specific projections and connections. How multiple subtypes of

enteric neurons are generated during development is a central problem in enteric developmental neurobiology. Most enteric neurons and glial cells arise from neural crest cells that emigrate from the caudal hindbrain ('vagal' level of the neural axis), but sacral neural crest cells contribute some neurons and glial cells, mostly to the hindgut [43–47].

Developmental appearance and birthdating of enteric neuron subtypes and glial cells

In this review, 'developmental appearance' is defined as the earliest developmental age at which expression of specific pan-neuronal or neuron subtype-specific markers can be localized to neural crest-derived cells in the gut; 'cell cycle exit' is defined as the time at which an enteric neuron precursor undergoes its last cell division; and 'birthdate' is defined as the developmental age at which a neuronal precursor exits the cell cycle.

Developmental appearance of pan-neuronal markers and enteric neuron subtypes

In the mouse, neural crest cells emigrate from the caudal hindbrain around E8.5, migrate ventrally and enter the foregut around E9.5–E10 [48]. They then migrate caudally along the gut and reach the end of the hindgut around E14.5 [49, 50]. Pan-neuronal markers (molecular characteristics shared by all neurons) are not expressed by vagal neural crest cells en route to the foregut [48]. But, by E10–10.5, around 10–20% of neural crest-derived cells in the stomach and rostral small intestine have already begun to express pan-neuronal markers including Hu, neuron class III β-tubulin (Tuj1), neurofilament-M and PGP9.5 [51, 52]. In both mice and chick embryos, cells expressing pan-neuronal markers are present close to the migratory wavefront [52–55] (Fig. 1B). A recent time-lapse imaging study showed that some cells expressing pan-neuronal markers continue to migrate caudally along the gut in close association with undifferentiated crest-derived cells [56]. This is not surprising as it is well established that immature neurons in many parts of the CNS migrate, some for considerable distances [57].

As in most other parts of the nervous system, different enteric neuronal subtypes begin to differentiate at different developmental stages [58–60]. Some enteric neuron subtype-specific markers are expressed early during the development of the mouse ENS. For example, in the ENS of E11.5 mice, some neurons show calbindin or NOS immunoreactivity, or express *Cart* (cocaine- and amphetamine-regulated transcript), and some of the neurites show immunoreactivity to the intermediate conductance potassium channel IK_{Ca}- [61, 62] (Fig. 1C). In the mature ENS, VIP is co-localized with NOS in enteric neurons, but during development, VIP immunoreactivity cannot be detected until several days after the appearance of NOS [63]. Thus there is a staggered appearance of markers of neuronal subtypes during development.

Acetylcholine is a major excitatory neurotransmitter at neuro-neuronal and neuromuscular junctions in the mature ENS. Using the synthesis of ^{3}H-acetylcholine from ^{3}H-choline as a marker, cholinergic neurons were first detected from E10 to E12 [58], although immunoreactivity to ChAT and vesicular acetylcholine transporter (VAChT) cannot be detected until around E18.5, possibly because these antisera are not very sensitive. ChAT-immunoreactive neurons are already present in the myenteric plexus of the small intestine of E18 rats, but earlier stages were not examined [64]. In embryonic mice, neuropeptide Y (NPY) can be first detected at E13–13.5 [65], substance P at E14–14.5 [63] and calcitonin gene-related peptide (CGRP), which is expressed by intrinsic sensory neurons [66], can be first detected at E17–17.5 [65]. In both mice and rats, some enteric neuron subtypes do not appear until after birth, and thus maturation of the ENS is thought to continue for several weeks after birth [60, 64, 67, 68].

In the zebrafish gut, cells expressing pan-neuronal markers appear between 24 and 48 hrs post-fertilisation (d.p.f.), beginning with the appearance of a few Hu-immunoreactive cells in the proximal gut [30, 69]. By 3 d.p.f, neurons are present along the entire length of the gut [30, 69, 70]. As in mice, it appears that the earliest developing neuron subtype is nitrergic neurons, with NOS immunoreactivity identified in the proximal midgut at 2 d.p.f. [28, 71, 72]. *In situ* hybridization studies also show the expression of nNOS mRNA in the gut between 2 and 3 d.p.f. [73, 74]. By 3 d.p.f., NOS neurons are present along most of the gut [71, 72]. VIP, pituitary adenylate cyclase activating peptide (PACAP), calbindin, calretinin and neurokinin A-immunoreactive neurons are also present along the entire gut at 3 d.p.f., and 5-HT and CGRP-immunoreactive neurons can be identified at 3–4 d.p.f. [70, 72]. ChAT-immunoreactivity could not be detected during zebrafish development, even though it is readily identifiable in the adult zebrafish ENS [72]. This is similar to the situation in embryonic mice, where ChAT-immunoreactive neurons cannot be detected until late embryonic stages (see above).

The development of nerve terminals within various target tissues innervated by enteric neurons has been examined in some species. In the mouse, NOS-containing nerve fibres are present in the circular muscle 2 days prior to birth (Fig. 1D, E), but cholinergic (VAChT-immunoreactive) nerve terminals are relatively sparse in the colon at birth; the densities of both NOS and VAChT terminals in the colonic circular muscle increase dramatically between P0 and P10 [75]. However, there is some functional evidence for a cholinergic innervation of the circular muscle in the small intestine of embryonic mice as excitatory junction potentials can be evoked by electrical field stimulation of E17 mice [76]. The innervation of the mucosa in the small and large intestine of the pig develops around birth [77], whereas in the mouse, nerve terminals are present in the mucosa in the small intestine at E18.5, just prior to birth [78].

In the developing nervous system, some neurotransmitters or their synthesizing enzymes, or combinations of neurotransmitters,

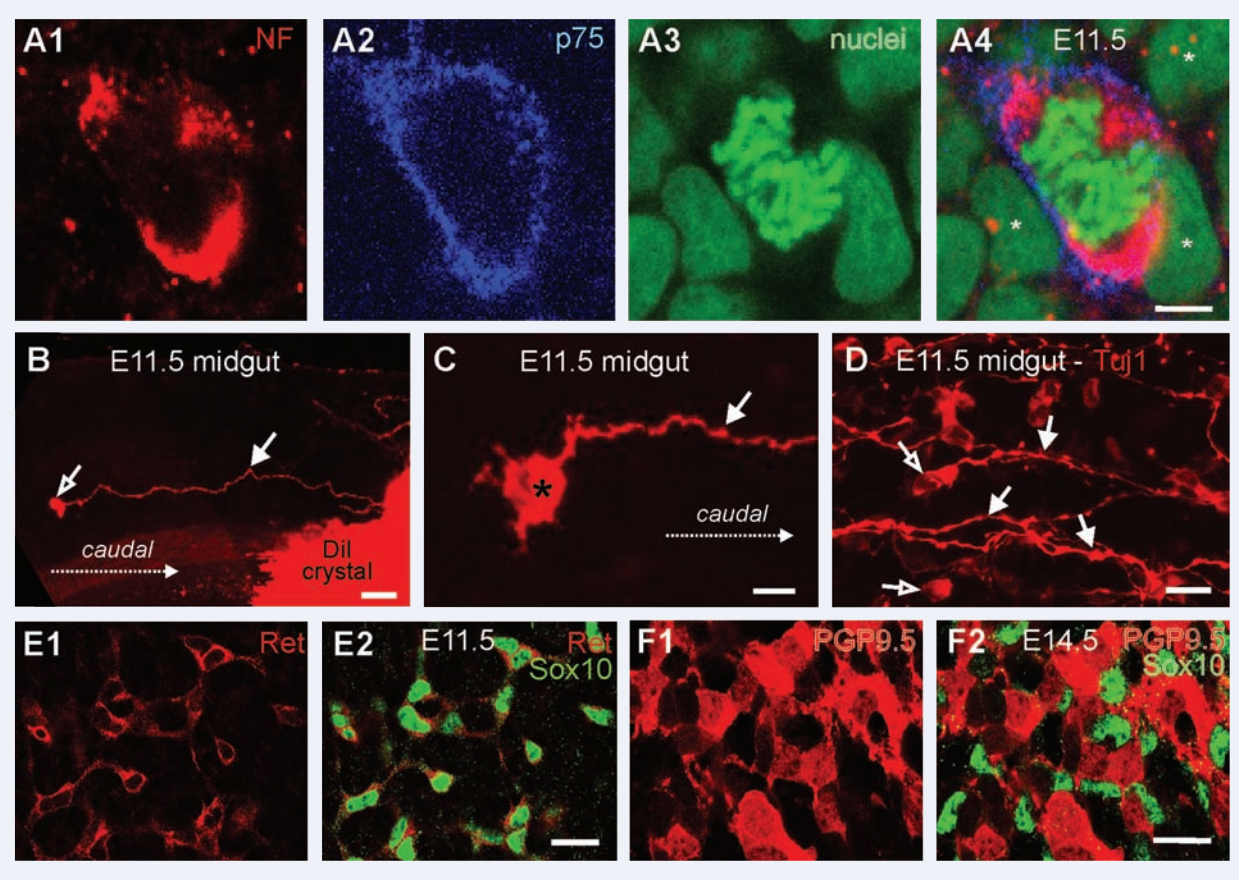

Fig. 2 (**A1–4**) A dividing immature neuron in a preparation of E11.5 midgut immunostained performed with the pan-neural crest cell marker, p75 (*blue*) and the pan-neuronal marker, neurofilament-M (*red–NF*). The nuclei had been stained using the nucleic acid stain, SYTO (*green*). The immature neuron is undergoing mitosis as the chromosomes are condensed (**A3**) Cells that do not show p75 immunostaining (*asterisks*) are mesenchymal cells. (**B**) Low magnification image of a cell body (*open arrow*) and axon (*arrow*) retrogradely labelled by the lipophilic dye, DiI, in the midgut from an E11.5 mouse. The neuron projects caudally. (**C**) Higher magnification image of a caudally projecting neuron in the E11.5 gut with a single axon (*arrow*). The neuron possesses several short, filamentous dendrites. (**D**) Preparation of midgut from an E11.5 mouse immunostained performed with antibodies to the pan-neuronal marker, Tuj1. Most of the neurites (*arrows*) are varicose and project longitudinally. Tuj1 cell bodies (*open arrows*) are scattered along the gut. (**E**) E11.5 gut immunostained with Ret (*red*) and Sox10 (*green*) antibodies. Most Ret^{+} cells also express Sox10. (**F**) E14.5 small intestine immunostained with PGP9.5 and Sox10. Sox10 is not expressed by $PGP9.5^{+}$ cells. Scale bars: A = 5 μm; B = 100 μm; C = 10 μm; D, E, F = 25 μm.

which are not expressed in the mature nervous system, are expressed transiently [79, 80]. In the developing ENS, the catecholamine synthetic enzyme, tyrosine hydroxylase (TH), is transiently expressed by all developing enteric neurons from E9.5 to E12.5 [51, 52, 56, 81, 82]. Signals arising from the gut appear to be important in the down-regulation of TH expression by immature enteric neurons [83]. A very small population of TH neurons (<0.5% of myenteric neurons) is present in the adult mouse [11], but these do not appear to arise from the transiently TH cells that are present early in embryonic development [84]. Although NOS is only expressed by a very small percentage of submucosal neurons in the small intestine [19], and the mucosa is not innervated by NOS nerve fibres in most mammals [85], in late embryonic and early post-natal mice, nearly 50% of submucosal neurons transiently express NOS (Fig. 1F), and NOS nerve fibres are present transiently in the mucosa [78].

Time of exit from cell cycle of different neuron types

The stage at which a neuronal precursor exits the cell cycle is defined as the birthdate of the neuron. In the CNS, neuronal precursors are generally thought to exit the cell cycle prior to expressing pan-neuronal markers. However, like developing sympathetic neurons, neural crest-derived cells continue to divide after expressing pan-neuronal markers during enteric neurogenesis [81, 86] (Fig. 2A). To

Table 1 The earliest detection of subtype-specific markers, birthdates and the peak of births of myenteric neuron subtypes in the mouse small intestine

Enteric neuron subtype	Earliest detection	Birthdates (myenteric plexus)	Peak of birth (myenteric)	References
5-HT	E18*	E8–E14	E10	[58, 88]
Cholinergic	E11†	E8–E15	E12	[58, 88]
Calbindin	E11.5	E12.5–P1‡	E14.5	[62, 89]
CART (cocaine- and amphetamine-regulated transcript)	E11.5	?	?	[61]
Calretinin	P0	?	?	[68]
CGRP	E17–17.5	E10–P3	E17	[65, 88]
Enkephalin	?	E10–E18	E14	[88]
GABA	?	E12.5–P1‡	E14.5	[89]
NOS	E11.5	E12.5–P1‡	E14.5	[62, 89]
NPY	E13–E13.5	E10–E18	E15	[65, 88]
Substance P	E14.5	?	?	[63]
VIP	E13.5	E10–P5	E15	[65, 88]

*Neurites in the mouse duodenum at E12 are able to take up radioactively labeled 5-HT. However, immunoreactivity to 5-HT in neurons is not present until E18 in the myenteric plexus [58].
†Cholinergic neuron differentiation identified by the synthesis of radioactively labeled ACh from ^{3}H-choline, not by immunohistochemistry. It is important to note that multiple enteric neuron subtypes are cholinergic, and different cholinergic neuron subtypes are likely to be born at different developmental stages.
‡Represents entire period of birthdating examined; birth of NOS and calbindin neurons very likely occurs prior to E12.5, as NOS and calbindin neurons are already present at E11.5.
? not yet examined.

date, there are no reports of mammalian enteric neurons undergoing cell division after the expression of neuron subtype-specific markers, so exit from the cell cycle appears to occur after the expression of pan-neuronal markers but prior to the expression of subtype-specific markers [87].

Only two studies have examined the birthdates of enteric neurons [88, 89]. In these studies in mouse, radiolabelled thymidine or thymidine analogues were injected into pregnant mice carrying embryos at different development stages [88, 89]. The 5-HT and cholinergic neurons are born first at very early ages, from E8 to E14, and E8 to E15, respectively, and myenteric VIP, NPY, enkephalin and CGRP neurons begin to be born at E10 [88]. A subsequent study showed that NOS, calbindin and γ-amino butyric acid (GABA)ergic neurons were born from E12.5 to P1, although younger birthdates were not investigated in this study [89]. Submucosal neurons tend to be born later than myenteric plexus neurons [88, 89]. In chick embryos, VIP neurons are born between E3 and E10, with a peak at E7, and VIP immunoreactivity is first detectable at E5.5–E6.5 [90].

Overall, there does not appear to be any obvious correlation between the birthdates of different types of neurons and the developmental stage at which they express specific markers at detectable levels for the first time (Table 1). For example, although the birth of 5-HT neurons peaks at E10 [88], endogenous production of 5-HT is not detectable until E18 [58]. Conversely, NOS- and calbindin-immunoreactive neurons can be identified at E11.5, and the peak of their births occurs at E14.5 [89]. Thus, the length of time between cell cycle exit and detectable expression of neuron subtype-specific markers differs for different enteric neuron subtypes.

Morphological development of enteric neurons

In the guinea-pig and mouse, some subtypes of enteric neurons have distinctive morphologies. For example, intrinsic sensory neurons are thought to be the only neurons with Dogiel type II morphology [91, 92]. However, other morphologies, such as Dogiel type I, are shared by multiple subtypes of neurons [7, 11]. The development of neuronal morphology in the embryonic mouse intestine *in vivo* has been examined using the lipophilic dye, DiI [53]. A caveat of this study is, however, that because of the size of

the DiI crystals used relative to the diameter of the gut, neurons that projected short distances locally or circumferentially would not have been detected. All of the neurons retrogradely labelled with DiI in the E11.5–E16.5 mouse gut had a single long process, presumably the axon, and multiple short filamentous processes, which are presumably dendrites [53] (Fig. 2B, C). A recent study of horizontal cells in the retina has shown that vertical neurites appear transiently, which lack synapses, but appear to be important for the regular spacing of horizontal cells; later in development, the vertical neurites are retracted and replaced by lateral dendrites [93]. In the mature ENS, all NOS neurons have Dogiel type I morphology, which are characterized by short lamellar dendrites [11], and many of the synapses occur onto the lamellar dendrites [94]. However, all of the DiI-labelled, immature NOS neurons in the embryonic mouse gut were reported to possess filamentous neurites [53]. It remains to be determined whether the filamentous neurites on immature NOS neurons receive synapses and/or play another role, and how the lamellar dendrites of NOS neurons form.

Some of the mechanisms controlling the development of enteric neuron morphology were examined in a study of neural crest-derived cells isolated from the gut of embryonic rats and grown in culture [95]. Pharmacological inhibition of the serine/threonine kinase, glycogen synthase kinase 3 beta (GSK3β) or protein kinase C zeta resulted in a significant increase in the proportion of neurons with multiple axons. It is therefore possible that enteric neurons destined to become multi-axonal Dogiel type II neurons express lower levels of GSK3β and protein kinase C zeta than other neuron subtypes.

Retinoid receptors and biosynthetic enzymes are expressed in the vicinity of crest-derived cells in the developing gut [96]. Retinoic acid reduces the length of neurites that extend from cultured crest-derived cells, possibly through an indirect effect on RhoA [96], and thus it is likely that retinoic acid influences enteric neuron morphology *in vivo*.

The mechanisms of morphological maturation of enteric neurons are still poorly understood. Although the developmental appearance of some neurofilaments and microtubule-associated proteins in enteric neurons has been examined [97], detailed studies of the mechanisms underlying the expression and intracellular localization of cytoskeleton components and regulators of cytoskeletal assembly and turnover during development of the ENS are required.

Axon guidance in the developing ENS

Different subtypes of enteric neurons differ in the targets they innervate (myenteric neurons, submucosal neurons, longitudinal muscle, circular muscle, mucosa, blood vessels) and the direction that their axons project. For example, most intrinsic sensory neurons have multiple axons that project circumferentially around the gut and to the mucosa, excitatory circular muscle motor neurons project orally and inhibitory circular muscle motor neurons project anally [1].

Some neural guidance cues have been shown to play a role in the migration of enteric neural crest-derived cells. For example, netrins/deleted in colorectal cancer (DCC) are essential for the centripetal migration of cells from the myenteric plexus to form the submucous plexus [98], and semaphorin 3A regulates the time of entry of sacral neural crest cells into the distal hindgut [99, 100]. However, the mechanisms controlling the navigation of the axons of developing enteric neurons to their correct targets and in the correct direction (up, down or around the gut) are largely unknown. To date there have been no enteric axon targeting defects reported in mice lacking any of the major neural guidance molecules, although it is unlikely that the ENS in any of these mice has been analysed with methods that would detect defects in axonal projections to targets. To our knowledge, the only mutant mouse with a defect in enteric axons are mice lacking the neurotrophic factor, neurturin, or its binding partner, GDNF family receptor alpha 2 (GFRα2); these mice have a reduced density of excitatory axons in the circular muscle (see below).

Many of the first enteric neurons to develop have a long leading axon-like process that projects anally in close association with migrating crest-derived cells [53, 101] (Fig. 1B). Experiments performed with DiI as a retrograde tracer also show that most immature enteric neurons project anally (Fig. 2B, C). Some of the immature anally projecting neurons express NOS (Fig. 1C). Using either pan-neuronal immunostaining or DiI tracing, prominent longitudinally projecting fibres (along the rostro-caudal axis of the gut) are observed shortly after each gut region is colonized by crest-derived cells (Fig. 2D). Therefore, there must be cues inducing a majority of neurites of the first enteric neurons to project longitudinally along the rostro-caudal axis of the gut. It has been proposed that the direction of axon projections of the first enteric neurons is influenced by the caudal direction of neural crest cell migration [53], although it is possible that other cues contribute to the projection of neurites along the rostro-caudal axis. The mechanisms inducing sub-populations of developing enteric neurons to project orally, circumferentially or to navigate to specific targets at later developmental stages remain to be elucidated.

Development of connectivity

The ENS is capable of functioning autonomously because of the existence of complex circuitry within the gut wall. Electron microscopic, confocal microscopic, physiological and pharmacological studies have led to the identification of the neuronal circuitry underlying some gut functions including motility [1, 18, 102], but little is known about how this circuitry develops. Immature neurons develop early, while the gut is being colonized by neural crest-derived cells (see above), and most have a long axon-like leading process that projects anally and is closely associated with other neural crest-derived cells and immature neurons [53, 56, 103]. The axon-like leading processes have prominent varicosities along their length (Fig. 2D). Varicosities in the embryonic gut

show synaptophysin immunoreactivity [104], and some synaptic proteins, including synaptotagmin 1 and synaptosome – associated protein of 25 000 daltons (SNAP25), were found to be differentially expressed in screens of gene expression comparing RNA from the intestine of E14.5 or E15.5 wild-type mice and mutant mice lacking enteric neurons [61, 105]. Hence at least some synaptic proteins are expressed in the developing ENS shortly after the gut is colonized by neural crest-derived cells.

There have been few ultrastructural studies of the developing ENS. The membranes of neurites were shown to be in direct apposition to the membranes of neural crest-derived cells in gut from E11 to E13.5 mice [106]. In a later detailed ultrastructural study, the swollen region of axons in the gut of E12.5 mice were shown to contain a small number of dispersed large granular vesicles and to form immature synapses with immature neurons that were characterized by thickened post-synaptic membranes [104]. By E16.5, more mature synapses were observed onto neuronal cell bodies; the pre-synaptic elements contained many vesicles, both small clear vesicles and large granular vesicles, which were clustered at the pre-synaptic membrane [104]. By E18.5, most of the contacts between axons and the myenteric nerve cell bodies had the features of mature synapses including clustered vesicles and thickened pre- and post-synaptic membranes [104]. Thus the anatomical substrates for synaptic transmission between myenteric neurons are in place well prior to birth in the mouse.

To date there have been no electrophysiological studies of enteric neurons in embryonic animals to determine whether immature neurons are spontaneously active and when synaptic inputs first develop.

Mechanisms controlling enteric neuronal differentiation and the generation of neuron diversity

During development, each neural stem/progenitor cell must first decide whether to adopt a neuronal or a glial cell fate. In most regions of the developing nervous system, neural stem cells initially give rise to committed neuronal precursors and only later to glial precursors [107]. It is also likely that the output of neural stem/progenitor cells changes over time.

Although studies carried out in the past few decades have provided extensive knowledge about the types of neurons present in the ENS and their connectivity [1,102], little is known about the mechanisms controlling the development of enteric neuron subtypes. Progenitor cells in the mouse ENS must generate at least 11 subtypes of myenteric neurons, five submucosal neuron subtypes, plus glial cells [11, 19]. Within each myenteric ganglion, neurons of a particular subtype do not appear to be clonal [50].

In some parts of the CNS, various subtypes of neurons are generated a long distance from their final destination, which permits physical segregation of inductive cues. For example, in the developing cerebral cortex, glutaminergic neurons are generated locally whereas GABAergic interneurons are generated in the ganglionic eminence and then migrate into the cortex. However, in the ENS different neuron subtypes are generated in the same environment. It therefore seems most likely that local or intrinsic cues regulate the development of enteric neuron subtypes.

Transcriptional control of enteric neuronal differentiation and the generation of neuron diversity

A number of different transcription factors are required for the development of the ENS including Phox2b, Sox10, Ascl1 (also called Mash1), Pax3, Hand2 and Hlx [108]. Some are required for the survival of neurons (Hand2) or glial (Sox10) progenitors after lineage segregation (see below), but only one transcription factor, Ascl1, has been associated with the development of a specific subtype of enteric neuron. *Ascl1*$^{-/-}$ mice lack enteric neurons in the esophagus and die at birth; importantly, they also lack 5-HT neurons in the intestines [109]. However, as around 50% of crest-derived cells in the embryonic mouse gut express Ascl1 [110] but 5-HT neurons comprise only around 1% of myenteric neurons in the mature ENS [11], it appears that not all Ascl1-expressing crest-derived cells are destined to become 5-HT neurons.

Sox10 is first expressed by neural crest cells as they delaminate from the neural tube and appears to be required for the survival and maintenance of neural crest-derived cells prior to lineage segregation, and then later for the development of the glial lineage [111–114]. Sox10 is down-regulated as cells differentiate into neurons [115] (Fig. 2E, F). After crest-derived cells arrive in the gut, a sub-population starts to express Ascl1, which has been proposed to suppress Sox10 expression; the Ascl1 crest cells are thought to give rise to neurons, while the Sox10 cells give rise to further progenitors or to glia [110] (Fig. 3).

Hand2 appears to be involved in the differentiation of enteric neurons, but not glial cells [116] (Fig. 3). Mice lacking Hand2 die at E10.5 from cardiovascular defects. However, if explants of gut are removed from E9.5 *Hand2*$^{-/-}$ mice and cultured for 6–10 days, markers of undifferentiated crest-derived cells including Sox10, p75, Ret and Phox2b, as well as glial precursors, identified by the expression of brain-derived fatty acid binding protein (B-FABP) are present, but cells expressing pan-neuronal markers are absent [116]. Two studies have examined embryonic mice in which there is a targeted deletion of Hand2 from neural crest cells, which die at E12.5–E16.5 [116, 117]. Although cells expressing some pan-neuronal markers are present in the gut of these embryonic mice, neuron subtype-specific markers, such as NOS or VIP are absent [116, 117], leading to the suggestion that Hand2 is required for the terminal differentiation of enteric neurons [116].

In mice lacking Phox2b, Sox10, Pax3 or Hlx, neural crest-derived cells fail to colonize most of the gastrointestinal tract, due to an essential role for these factors in the early survival of enteric

crest-derived cells [108, 111, 114, 118, 119]. In some parts of the developing nervous system it has been shown that a single transcription factor can be involved in early events in the formation of the nervous system, and then be involved in later events including neuronal specification and neuronal migration [120, 121]. It is therefore possible that Phox2b, Pax3 and Hlx might also play later roles in the development of the ENS, including subtype specification, which is likely only to be revealed by conditional knockout of the genes at specific developmental stages. In adult mice, Phox2b is expressed by enteric neurons and glia, whereas Sox10 is only expressed by enteric glia [2, 115, 122, 123].

A major mechanism in the generation of neuronal diversity in many regions of the CNS is that of 'combinatorial codes'. For example, a combination of five transcription factors, including Ascl1, is involved in the differentiation of 5-HT neurons in the mouse hindbrain, although Ascl1 appears to be 'an essential and general determinant of the 5-HT phenotype' [124], including 5-HT neuron differentiation in the ENS (see above). Although there do not appear to be universal genetic programmes controlling the development of neurons with a particular neurotransmitter phenotype in different regions of the nervous system [125], a recent study showed that the genes that encode proteins for dopamine synthesis and transport (dopaminergic terminal selector genes) share an evolutionary conserved DNA regulatory sequence [126]. It therefore appears that in different regions of the nervous system, different transcriptional networks can be involved in the activation of the same terminal selector genes. Transcription factors that specify neurotransmitter identity often also control other features of the neuronal phenotype, such as axon pathfinding [127]. To date, transcriptional codes involved in the development of subtypes of enteric neurons have not been identified.

Role of glial cell line-derived neurotrophic factor (GDNF) family members in enteric neuronal differentiation and the generation of neuron diversity

The most important GDNF family member involved in ENS development is GDNF [29, 128–134]. GDNF is required for the survival of enteric neural crest-derived cells, as neural crest cells die around E9.5, just as they are entering the foregut, in mice lacking GDNF or its signaling receptor, Ret [135]; *Gdnf*$^{-/-}$ and *Ret*$^{-/-}$ mice die at birth [128, 136–138]. GDNF is expressed by the gut mesenchyme [139, 140] and Ret by enteric neural crest cells [141]. Because of its essential role in the early survival of crest-derived cells, it has not yet been possible to study any possible roles for GDNF in the later development of the ENS *in vivo*. However, studies *in vitro* have shown that GDNF promotes the proliferation, migration and neuronal differentiation of enteric neural crest-derived cells, as well as survival [129, 142–146]. In adult *Gdnf*$^{+/-}$ mice there is over a 50% reduction in the number of myenteric neurons in the small intestine and colon [147, 148].

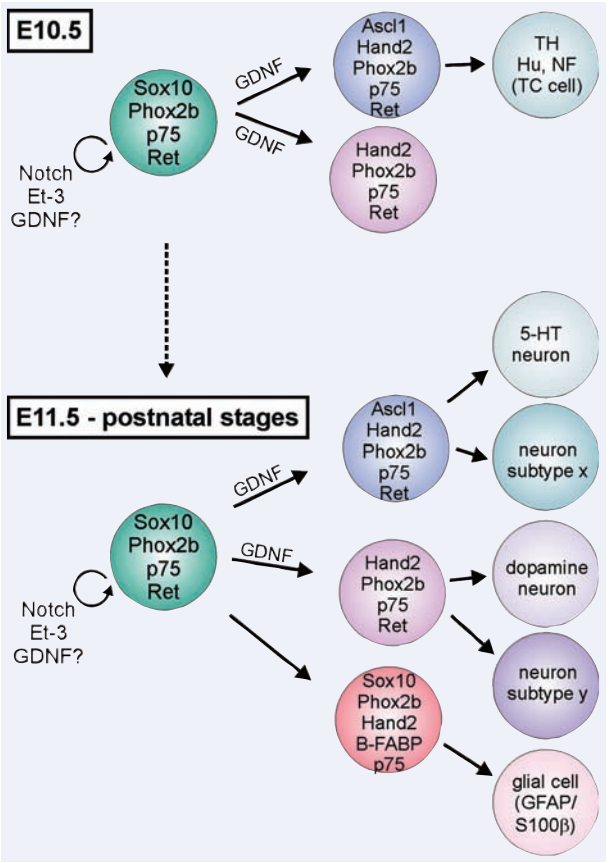

Fig. 3 Differentiation of enteric neurons and glial cells. At E10.5, the progeny of Sox10^{+}/Phox2b^{+}/p75^{+}/Ret^{+} neural progenitors are further self-renewing cells plus neuron precursors, which express Hand2 ± Ascl1. From E11.5 onwards, glial precursors are also generated, and neuron subtype expression commences. GDNF promotes the proliferation of enteric neural crest-derived cells, but it is unclear whether it only promotes the proliferation of neuronal precursors, or all progenitors. It is likely that the competence of Sox10^{+}/Phox2b^{+}/p75^{+}/Ret^{+} cells changes with age. TC, transiently catecholaminergic; TH, tyrosine hydroxylase. Data from [84, 109, 110, 115, 116, 143, 145, 147, 156].

The percentage of myenteric neurons expressing NOS is the same in *Gdnf*$^{+/-}$ mice as in wild-type mice, and thus NOS neurons are not selectively affected [148], although it remains possible that other neuron subtypes are selectively affected by *Gdnf* haploinsufficiency.

Mice lacking another GDNF family member, neurturin or its signaling co-receptor, GFRα2, are viable and have similar numbers of enteric neurons to wild-type mice [9, 10, 147, 149]. However, there is a decrease in the number of substance P (excitatory) axons innervating the circular muscle [9, 10, 149]. Neurturin, therefore, appears to be important in attracting the axons of excitatory motor neurons into the circular muscle and/or promoting axon growth and branching.

Role of endothelin-3/Ednrb signalling

Endothelin-3 is expressed by the gut mesenchyme [150,151] and its receptor, endothelin receptor B (Ednrb) is expressed by migrating neural crest cells and also by the gut mesenchyme [150, 152, 153]. In mice and rats lacking endothelin 3 or Ednrb, enteric neurons are missing from the distal regions of the gastrointestinal tract [154, 155].

There is considerable evidence from both *in vitro* and *in vivo* studies that the main function of endothelin-3 signalling in ENS development is to inhibit the rate of neuronal differentiation of enteric neural crest-derived cells (Fig. 3); in the absence of endothelin-3 signalling there is thought to be premature neuronal differentiation and a resultant smaller pool of undifferentiated cells, which are likely to be more proliferative and migratory than immature neurons [143, 156–158]. NOS neurons show a rostral shift along the gastrointestinal tract in their first appearance in *Et-3*$^{-/-}$ mice compared to wild-type mice, which was ascribed to an effect on migration rather than differentiation [159]. However, studies of post-natal and adult mice lacking endothelin-3 suggest that endothelin-3 signalling may also have subtle effects on the phenotype of specific sub-populations of neurons. For example, in P10 and adult *Et-3*$^{-/-}$ mice, NOS neurons comprise a significantly higher proportion of myenteric neurons rostral to the aganglionic region than in equivalent regions of wild-type mice [148, 160]. There are also differences in the expression of neurotransmitters in the small intestine of adult *Et-3*$^{-/-}$ mice [160].

Role of other signalling pathways

Neurotrophin-3 (NT-3)

NT-3 is the only neurotrophin that has been shown to influence enteric neuron development [161]. Mice lacking NT-3 or its preferred receptor, TrkC, show reductions in myenteric and submucosal neuron numbers, but some neuron subtypes are affected more than others, with a significant reduction in CGRP neurons, but not 5-HT, NOS, GABA, ChAT or substance P neurons [162].

Bone morphogenetic proteins (BMPs)

Studies *in vitro* have shown that BMPs influence multiple processes including crest cell migration, neuronal differentiation, fasciculation and cell aggregation [163–167] and many of the effects are concentration-dependent [167]. The role of BMPs in enteric neuron development *in vivo* has been examined using mice in which noggin is overexpressed in neurons using the neuron-specific enolase (NSE) promoter [167]. Mice overexpressing noggin have a higher density of enteric neurons, showing that BMPs regulate enteric neuron number [89, 167]. However, not all subtypes of neurons are affected by noggin overexpression; in the myenteric plexus, the density of serotonin, calretinin and calbindin neurons is increased, NOS and GABA neurons are unaffected, and the number of CGRP neurons is decreased [89]. As serotonin, calretinin and calbindin exit the cell cycle relatively early and CGRP neurons exit the cell cycle late, the effects of noggin overexpression are correlated with cell cycle exit [89], although it is unclear whether BMPs normally selectively influence the differentiation of specific neuron subtypes, or whether the differential effects of noggin overexpression on different neuron subtypes in the NSE-noggin transgenic mouse is related to the developmental stage at which noggin overexpression becomes functionally significant.

L1

The cell adhesion molecule, L1, influences the migration of enteric crest-derived cells [168]. A recent study has shown that L1 also influences the rate of neuronal differentiation, as in mice lacking L1 there is a delay in neuronal differentiation (expression of the pan-neuronal marker, Hu) and a delay in the differentiation of CGRP neurons, but not NOS neurons; these delays are rectified at later developmental stages [169]. The rate of glial differentiation is unaffected by loss of L1 [169].

Sonic hedgehog

Sonic hedgehog inhibits the neuronal differentiation of crest-derived cells from embryonic mice *in vitro* [170]. It is possible, therefore, that sonic hedgehog modulates the rate of neuronal differentiation *in vivo*, although the only reported defect in mice lacking sonic hedgehog is the presence of neurons in ectopic locations, such as the gut mucosa [171]. In zebrafish, sonic hedgehog is required for the migration of neural crest from the hindbrain into the anterior gut [172].

Role of electrical activity

In the developing spinal cord, endogenous GABA and glutamate release regulate the neurotransmitter phenotype of neurons [173]. During development of the ENS, it is possible that some enteric neurons become electrically active while other crest-derived cells are still differentiating. It is currently unknown when developing enteric neurons first become electrically active. However, a study of cultured neurons isolated from the embryonic rat gut showed that depolarization changed the phenotype of the neurons, and caused an increase in the proportion of VIP and TH neurons [174]. It therefore remains a possibility that electrical activity and spontaneous release of neurotransmitters from early differentiating neurons influence the neurotransmitter phenotype of later developing enteric neurons.

Development of enteric glia

Enteric glial cells are closely associated with enteric nerve cell bodies and their axons (Fig. 1A). Enteric glia plays an important role in intestinal epithelial cell function and in immune responses [175]. Ablation of enteric glia results in changes to enteric neurons including degeneration and changes in neurotransmitter

expression [176, 177]. Furthermore, recent *in vitro* and *in vivo* studies have demonstrated neuron-glial cell communication, as glial cells respond with an increase in intracellular Ca^{2+} to electrical stimulation or depolarization of enteric neurons [178, 179]. It is possible that glial cells are involved in some gastrointestinal motor and inflammatory disorders [180].

During development of the ENS there appears to be differences between species in the time at which enteric glia are generated. In the embryonic chick, glial fibrillary acidic protein (GFAP)-immunoreactive cells can be detected as neural crest-derived cells are colonizing the gut and some are present close to the migratory wavefront [54]. In contrast, in embryonic mice, GFAP cannot be detected until late embryonic stages [181], although glial precursors, identified by the expression of B-FABP [182], can be first detected about 24 hrs after each region is first colonized by neural crest-derived cells [115].

Little is known about the mechanisms controlling the development of enteric glial cells from neural crest stem/progenitor cells. Like all peripheral glial cells, Sox10 is required for the development of enteric glial cells [112, 182] (see above). Notch signalling has been shown to promote the generation of glial cells from neural crest stem/progenitor cells in the peripheral nervous system [183, 184]. Two studies have examined the role of Notch signalling in the developing ENS by examining mice in which there are neural crest cell-specific defects in Notch signalling [110, 185]. One study concluded that Notch signalling promotes gliogenesis because the development of enteric glia was more severely affected than enteric neuron development by loss of Notch signalling [185]. However, a subsequent study concluded that Notch signalling was required for the maintenance of enteric neuronal progenitors (Fig. 3), as premature neurogenesis was observed in the ENS of mice with defects in Notch signalling in neural crest-derived cells [110].

Development of neurons and neuronal subtypes in the human ENS and clinical relevance

The human gut is colonized by crest-derived cells in a rostral-to-caudal wave between weeks 4 and 7 [186, 187]. As in laboratory mammals [98, 188], the submucosal plexus forms after the myenteric plexus from a centripetal migration from the myenteric plexus [186, 187]. Cells showing immunoreactivity to the pan-neuronal marker PGP9.5 and to the glial marker, S100β, were detected in the foregut at week 7 [186], calretinin and calbindin neurons are present by week 8 [32] and substance P by week 9 [189]. NOS neurons are already present at week 10 [190] and there is a relatively dense plexus of NOS nerve fibres in the circular muscle by mid-gestation stages [191,192]. VIP is present in a sub-population of myenteric neurons at week 18, but there is no overlap between NOS and VIP immunoreactivities [191]. GFAP-immunoreactive enteric glial cells are present by week 10 [190]. TH neurons were detected from week 24 [193].

Hirschsprung's disease

The best characterized defect in ENS development is Hirschsprung's disease (HSCR), which is caused by an absence of enteric neurons from variable lengths of the distal bowel [133]. Proximal to the aganglionic region, there is usually a transition zone of decreased enteric neuron density [194]. The current treatment for HSCR is to remove surgically the abnormal regions of bowel. Calretinin immunostaining appears to be an accurate method for diagnosing HSCR from rectal suction biopsies [195, 196]. Calretinin fibres are completely absent from the aganglionic zone [195, 196] and thus unlike some other diagnostic markers or stains (*e.g.* acetylcholinesterase histochemistry), calretinin does not appear to be expressed by nerve fibres in the human gut that arise from extrinsic sources, which makes diagnosis less equivocal.

Is the ganglionic segment of Hirschsprung's patients 'normal'?

Patients with HSCR commonly suffer from ongoing motility problems even following surgery. Studies of mouse models of HSCR have shown that there is a decrease in the density of myenteric neurons in the ganglionic regions of the colon, and also some changes in neurotransmitter expression in the small intestine [148, 160]. Moreover, reduced numbers of interstitial cells of Cajal (ICC) were reported proximal to the aganglionic region [160]. In human infants, a recent study showed a decrease in the density nerve cells in the myenteric plexus of the ganglionic region of colon of 12 post-operative patients with HSCR compared to colonic samples from seven 'control' patients ranging from 1-month-old to 12 years with non-ENS gastrointestinal disorders [197]. However, the density of neurons in the myenteric plexus or nerve fibres in the circular muscle in the ganglionic region does not appear to be predictive of clinical outcome [197]. There seems to be a very high degree of variability between HSCR patients in the density of ICC in the ganglionic region, and there is some evidence that an imbalance between ICC and neuron densities are correlated with a poor clinical outcome, rather than decreased densities of neurons only or ICC only [197, 198].

Other paediatric motility disorders

HSCR is the easiest paediatric enteric neuropathy to diagnose because of the complete absence of enteric neurons from affected regions. However, developmental defects in the ENS are likely to underlie other, more common, paediatric motility disorders [199–201]. In particular, it is likely that defects in the development

of specific subtypes of enteric neurons, or in the number of enteric neurons, underlies some motility disorders. A major problem with analysing biopsy specimens from infants and children with motility disorders is the dearth of specimens from age-matched controls – some data are available about 'normal' enteric neuron numbers [202], but very little is known about the proportions of neuron subtypes and densities of different fibres in the circular muscle in infants and children of different ages. Furthermore, defects in receptor or ion channel expression, or in connectivity of enteric neurons, will be extremely difficult to diagnose.

Defects in the development of subtypes of enteric neurons

In infants with congenital hypertrophic pyloric stenosis, the innervation of the pylorus by inhibitory (NOS) neurons is defective [203]. As NOS neurons are present in myenteric ganglia and the innervation of the longitudinal muscle of the pylorus by NOS fibres appears normal, the failure of the axons of NOS neurons to grow into or survive in the circular muscle of these patients might reflect defects in the circular muscle rather than NOS neurons. Some children with slow transit constipation have a deficit of substance P nerve fibres in the circular muscle [204, 205], but it is unknown whether this deficit is a result or a cause of the disease.

Defects in the number of enteric neurons

Mutant mice in which myenteric neuron density is increased [206] or decreased [148] by around 50% have motility defects, and thus enteric neurons must be generated in the correct numbers for normal gut function. It is unknown, however, whether smaller changes in enteric neuron density (*e.g.* 10–30% increase or decrease) also result in abnormal motility or other gut functions.

Multiple endocrine neoplasia type 2B (MEN 2B) is a rare hereditary syndrome associated with an activating mutation in RET [207]. Most patients have mucosal neuromas, pheochromocytomas and medullary thyroid carcinoma, and also commonly experience gastrointestinal motility problems including constipation and intestinal obstruction [208–210]. Grossly enlarged myenteric and submucosal ganglia (ganglioneuromatosis) are observed in biopsy specimens [208, 209]. An immunohistochemical study of colonic muscle specimens from three children with MEN 2B revealed normal density of NOS and VIP fibres, but moderate to severe loss of substance P fibres when compared to fibre densities in the adult colon [209]. The low number of substance P fibres in the muscle is surprising given the increased number of ganglion cells in these patients [209], and may reflect defects in the growth or branching of axons of substance P containing neurons, although it is also possible that it is a secondary defect.

A number of children who suffer from chronic constipation have been diagnosed with a very controversial condition called intestinal neuronal dysplasia type B (INDB) [211–213]. Some pathologists claim that INDB is associated with an increase in the number of neurons per submucosal ganglion and an increase in the number of submucosal neurons, although the diagnostic criteria have undergone a number of revisions over the years [214]. However, a number of pathologists have raised doubts as to whether INDB is a distinct clinical entity [215,216].

Unfortunately, the etiology of abnormal intestinal motility in the vast majority of affected infants is unclear [200], and is hampered by a lack of knowledge of the post-natal development of the ENS in healthy humans, and by deficiencies in our knowledge of the classification of enteric neurons and by agreed and specific markers for each enteric neuron subtype in humans.

Conclusions

Enormous advances have been made in the past decade in understanding the genetic, molecular and cellular mechanisms involved in the colonization of the gut by enteric neuron precursors [29, 130, 131, 133, 217, 218]. However, very little is known about the mechanisms underlying the generation of different subtypes of enteric neurons with correct projections and connectivity, and the generation of enteric glia. Scant knowledge exists of the transcriptional control of enteric neuron subtypes and of the molecular mechanisms underlying axon targeting and synapse formation in the developing ENS. Defects in these processes are likely to underlie some paediatric motility disorders. An understanding of the mechanisms underlying the generation of enteric neuron subtypes will be key information in the potential application of cell therapy for enteric neuropathies.

Acknowledgements

We thank John Furness and Colin Anderson for valuable comments on the manuscript, Jeff Milbrandt and Hideki Enomoto for the mice shown in Figure 1B, and Annette Bergner, Rachael Roberts and Kirsty Turner for assistance with the immunohistochemistry and DiI tracing shown in Figures 1 and 2. The authors' research is supported by a Discovery Grant from the Australian Research Council and a Project grant and Fellowship from the National Health and Medical Research Council of Australia.

References

1. **Furness JB.** The enteric nervous system. Oxford, UK: Blackwell; 2006.
2. **Hoff S, Zeller F, von Weyhern CW, *et al*.** Quantitative assessment of glial cells in the human and guinea pig enteric nervous system with an anti-Sox8/9/10 antibody. *J Comp Neurol.* 2008; 509: 356–71.
3. **Gershon MD.** The second brain. New York: Harper Collins; 1998.
4. **Timmermans JP, Hens J, Adriaensen D.** Outer submucous plexus: an intrinsic nerve

network involved in both secretory and motility processes in the intestine of large mammals and humans. *Anat Rec.* 2001; 262: 71–8.

5. **Costa M, Brookes SJ, Steele PA, *et al.*** Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience.* 1996; 75: 949–67.
6. **Brookes SJ.** Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec.* 2001; 262: 58–70.
7. **Furness JB.** Types of neurons in the enteric nervous system. *J Auton Nerv Syst.* 2000; 81: 87–96.
8. **Sang Q, Young HM.** Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tissue Res.* 1996; 284: 39–53.
9. **Heuckeroth RO, Enomoto H, Grider JR, *et al.*** Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron.* 1999; 22: 253–63.
10. **Rossi J, Herzig KH, Voikar V, *et al.*** Alimentary tract innervation deficits and dysfunction in mice lacking GDNF family receptor alpha2. *J Clin Invest.* 2003; 112: 707–16.
11. **Qu ZD, Thacker M, Castelucci P, *et al.*** Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res.* 2008; 334: 147–61.
12. **Chen JJ, Li Z, Pan H, *et al.*** Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: abnormal intestinal motility and the expression of cation transporters. *J Neurosci.* 2001; 21: 6348–61.
13. **Allen JP, Canty AJ, Schulz S, *et al.*** Identification of cells expressing somatostatin receptor 2 in the gastrointestinal tract of Sstr2 knockout/lacZ knockin mice. *J Comp Neurol.* 2002; 454: 329–40.
14. **Neal KB, Parry LJ, Bornstein JC.** Strain-specific genetics, anatomy and function of enteric neural serotonergic pathways in inbred mice. *J Physiol.* 2009; 587: 567–86.
15. **Sang Q, Williamson S, Young HM.** Projections of chemically identified myenteric neurons of the small and large intestine of the mouse. *J Anat.* 1997; 190: 209–22.
16. **Portbury AL, Pompolo S, Furness JB, *et al.*** Cholinergic, somatostatin-immunoreactive interneurons in the guinea pig intestine: morphology, ultrastructure, connections and projections. *J Anat.* 1995; 187: 303–21.
17. **Grider JR.** Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J Pharmacol Exp Ther.* 2003; 307: 460–7.
18. **Furness JB, Jones C, Nurgali K, *et al.*** Intrinsic primary afferent neurons and nerve circuits within the intestine. *Prog Neurobiol.* 2004; 72: 143–64.
19. **Mongardi FC, Thacker M, Chiocchetti R, Furness JB.** Identification of neuron types in the submucosal ganglia of the mouse ileum. *Cell Tissue Res.* 2009; 336: 179–89.
20. **Sundler F, Ekblad E, Hakanson R.** Projections of enteric peptide-containing neurons in the rat. *Arch Histol Cytol.* 1989; 52 Suppl: S181–9.
21. **Mann PT, Furness JB, Southwell BR.** Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. *Cell Tissue Res.* 1999; 297: 241–8.
22. **Timmermans JP, Barbiers M, Scheuermann DW, *et al.*** Distribution pattern, neurochemical features and projections of nitrergic neurons in the pig small intestine. *Ann Anat.* 1994; 176: 515–25.
23. **Hens J, Schrodl F, Brehmer A, *et al.*** Mucosal projections of enteric neurons in the porcine small intestine. *J Comp Neurol.* 2000; 421: 429–36.
24. **Brown DR, Timmermans JP.** Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil.* 2004; 16 Suppl 1: 50–4.
25. **Chiocchetti R, Grandis A, Bombardi C, *et al.*** Characterisation of neurons expressing calbindin immunoreactivity in the ileum of the unweaned and mature sheep. *Cell Tissue Res.* 2004; 318: 289–303.
26. **Mazzuoli G, Mazzoni M, Albanese V, *et al.*** Morphology and neurochemistry of descending and ascending myenteric plexus neurons of sheep ileum. *Anat Rec.* 2007; 290: 1480–91.
27. **Hens J, Gajda M, Scheuermann DW, *et al.*** The longitudinal smooth muscle layer of the pig small intestine is innervated by both myenteric and submucous neurons. *Histochem Cell Biol.* 2002; 117: 481–92.
28. **Olden T, Akhtar T, Beckman SA, *et al.*** Differentiation of the zebrafish enteric nervous system and intestinal smooth muscle. *Genesis.* 2008; 46: 484–98.
29. **Burzynski G, Shepherd IT, Enomoto H.** Genetic model system studies of the development of the enteric nervous system, gut motility and Hirschsprung's disease. *Neurogastroenterol Motil.* 2009; 21: 113–27.
30. **Holmberg A, Schwerte T, Fritsche R, *et al.*** Ontogeny of intestinal motility in correlation to neuronal development in zebrafish embryos and larvae. *J Fish Biol.* 2003; 63: 318–31.
31. **Wattchow DA, Furness JB, Costa M.** Distribution and coexistence of peptides in nerve fibers of the external muscle of the human gastrointestinal tract. *Gastroenterology.* 1988; 95: 32–41.
32. **Walters JR, Bishop AE, Facer P, *et al.*** Calretinin and calbindin-D28k immunoreactivity in the human gastrointestinal tract. *Gastroenterology.* 1993; 104: 1381–9.
33. **Matini P, Manneschi LI, Mayer B, *et al.*** Nitric oxide producing neurons in the human colon: an immunohistochemical and histoenzymatical study. *Neurosci Lett.* 1995; 193: 17–20.
34. **Porter AJ, Wattchow DA, Brookes SJ, *et al.*** The neurochemical coding and projections of circular muscle motor neurons in the human colon. *Gastroenterology.* 1997; 113: 1916–23.
35. **Wattchow DA, Porter AJ, Brookes SJ, *et al.*** The polarity of neurochemically defined myenteric neurons in the human colon. *Gastroenterology.* 1997; 113: 497–506.
36. **Manneschi LI, Vannucchi MG, Bechi P, *et al.*** Neuron density and distribution of NADPH-diaphorase positive neurons in the human stomach. *Neurosci Lett.* 1998; 250: 169–72.
37. **Brehmer A, Croner R, Dimmler A, *et al.*** Immunohistochemical characterization of putative primary afferent (sensory) myenteric neurons in human small intestine. *Auton Neurosci.* 2004; 112: 49–59.
38. **Brehmer A, Lindig TM, Schrodl F, *et al.*** Morphology of enkephalin-immunoreactive myenteric neurons in the human gut. *Histochem Cell Biol.* 2005; 123: 131–8.
39. **Faussone-Pellegrini MS, Taddei A, Bizzoco E, *et al.*** Distribution of the vanilloid (capsaicin) receptor type 1 in the human stomach. *Histochem Cell Biol.* 2005; 124: 61–8.
40. **Brehmer A, Schrodl F, Neuhuber W.** Morphology of VIP/nNOS-immunoreactive myenteric neurons in the human gut. *Histochem Cell Biol.* 2006; 125: 557–65.
41. **Murphy EM, Defontgalland D, Costa M, *et al.*** Quantification of subclasses of human colonic myenteric neurons by immunoreactivity to Hu, choline acetyltransferase and nitric oxide synthase. *Neurogastroenterol Motil.* 2007; 19: 126–34.
42. **Hens J, Vanderwinden JM, De Laet MH, *et al.*** Morphological and neurochemical

identification of enteric neurones with mucosal projections in the human small intestine. *J Neurochem.* 2001; 76: 464–71.
43. **Le Douarin NM, Teillet MA.** The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J Embryol Exp Morphol.* 1973; 30: 31–48.
44. **Yntema CL, Hammond WS.** The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J Comp Neurol.* 1954; 101: 515–41.
45. **Burns AJ, Le Douarin NM.** The sacral neural crest contributes neurons and glia to the post- umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. *Development.* 1998; 125: 4335–47.
46. **Druckenbrod NR, Epstein ML.** The pattern of neural crest advance in the cecum and colon. *Dev Biol.* 2005; 287: 125–33.
47. **Kapur RP.** Colonization of the murine hindgut by sacral crest-derived neural precursors: experimental support for an evolutionarily conserved model. *Dev Biol.* 2000; 227: 146–55.
48. **Anderson RB, Stewart AL, Young HM.** Phenotypes of neural-crest-derived cells in vagal and sacral pathways. *Cell Tissue Res.* 2006; 323: 11–25.
49. **Kapur RP, Yost C, Palmiter RD.** A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice. *Development.* 1992; 116: 167–75.
50. **Young HM, Newgreen D.** Enteric neural crest-derived cells: origin, identification, migration, and differentiation. *Anat Rec.* 2001; 262: 1–15.
51. **Baetge G, Gershon MD.** Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. *Dev Biol.* 1989; 132: 189–211.
52. **Young HM, Ciampoli D, Hsuan J, *et al.*** Expression of ret-, p75(NTR)-, Phox2a-, Phox2b-, and tyrosine hydroxylase-immunoreactivity by undifferentiated neural crest-derived cells and different classes of enteric neurons in the embryonic mouse gut. *Dev Dyn.* 1999; 216: 137–52.
53. **Young HM, Jones BR, McKeown SJ.** The projections of early enteric neurons are influenced by the direction of neural crest cell migration. *J Neurosci.* 2002; 22: 6005–18.
54. **Conner PJ, Focke PJ, Noden DM, *et al.*** Appearance of neurons and glia with respect to the wavefront during colonization of the avian gut by neural crest cells. *Dev Dyn.* 2003; 226: 91–8.
55. **Barlow AJ, Wallace AS, Thapar N, *et al.*** Critical numbers of neural crest cells are required in the pathways from the neural tube to the foregut to ensure complete enteric nervous system formation. *Development.* 2008; 135: 1681–91.
56. **Hao MM, Anderson RB, Kobayashi K, *et al.*** The migratory behavior of immature enteric neurons. *Dev Neurobiol.* 2009; 69: 22–35.
57. **Metin C, Vallee RB, Rakic P, *et al.*** Modes and mishaps of neuronal migration in the mammalian brain. *J Neurosci.* 2008; 28: 11746–52.
58. **Rothman TP, Gershon MD.** Phenotypic expression in the developing murine enteric nervous system. *J Neurosci.* 1982; 2: 381–93.
59. **Epstein ML, Hudis J, Dahl JL.** The development of peptidergic neurons in the foregut of the chick. *J Neurosci.* 1983; 3: 2431–47.
60. **Matini P, Mayer B, Faussone-Pellegrini MS.** Neurochemical differentiation of rat enteric neurons during pre- and postnatal life. *Cell Tissue Res.* 1997; 288: 11–23.
61. **Heanue TA, Pachnis V.** Expression profiling the developing mammalian enteric nervous system identifies marker and candidate Hirschsprung disease genes. *Proc Natl Acad Sci U S A.* 2006; 103: 6919–24.
62. **Hao MM, Moore RE, Anderson RB, *et al.*** Development of neurons and the role of neural activity in the developing gut. *Neurogastroenterol Motil.* 2009; 21: V-V. DOI: 10.1111/j.1365–2982.2008.01253.x
63. **Rothman TP, Nilaver G, Gershon MD.** Colonization of the developing murine enteric nervous system and subsequent phenotypic expression by the precursors of peptidergic neruons. *J Comp Neurol.* 1984; 225: 13–23.
64. **Vannucchi MG, Faussone-Pellegrini MS.** Differentiation of cholinergic cells in the rat gut during pre- and postnatal life. *Neurosci Lett.* 1996; 206: 105–8.
65. **Branchek TA, Gershon MD.** Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. *J Comp Neurol.* 1989; 285: 262–73.
66. **Furness JB, Robbins HL, Xiao J, *et al.*** Projections and chemistry of Dogiel type II neurons in the mouse colon. *Cell Tissue Res.* 2004; 317: 1–12.
67. **Vannucchi MG, De Giorgio R, Faussone-Pellegrini MS.** NK1 receptor expression in the interstitial cells of Cajal and neurons and tachykinins distribution in rat ileum during development. *J Comp Neurol.* 1997; 383: 153–62.
68. **Young HM, Torihashi S, Ciampoli D, *et al.*** Identification of neurons that express stem cell factor in the mouse small intestine. *Gastroenterology.* 1998; 115: 898–908.
69. **Bisgrove BW, Raible DW, Walter V, *et al.*** Expression of c-ret in the zebrafish embryo: potential roles in motoneuronal development. *J Neurobiol.* 1997; 33: 749–68.
70. **Olsson C, Holmberg A, Holmgren S.** Development of enteric and vagal innervation of the zebrafish (Danio rerio) gut. *J Comp Neurol.* 2008; 508: 756–70.
71. **Holmberg A, Olsson C, Holmgren S.** The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol.* 2006; 209: 2472–9.
72. **Uyttebroek L, Harrissoon F, Hubens G, *et al.*** Neurochemical identification of enteric neurons in the larval and adult intestine of the zebrafish *(Danio rerio). Neurogastroenterol Motil.* 2009; 21(2): xxiv-xxiv. DOI: 10.1111/j.1365–2982.2008.01253.x
73. **Poon KL, Richardson M, Lam CS, *et al.*** Expression pattern of neuronal nitric oxide synthase in embryonic zebrafish. *Gene Expr Patterns.* 2003; 3: 463–6.
74. **Holmqvist B, Ellingsen B, Forsell J, *et al.*** The early ontogeny of neuronal nitric oxide synthase systems in the zebrafish. *J Exp Biol.* 2004; 207: 923–35.
75. **Roberts RR, Murphy JF, Young HM, *et al.*** Development of colonic motility in the neonatal mouse-studies using spatiotemporal maps. *Am J Physiol Gastrointest Liver Physiol.* 2007; 292: G930–8.
76. **Ward SM, Harney SC, Bayguinov JR, *et al.*** Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis. *J Physiol.* 1997; 505: 241–58.
77. **Paran TS, Rolle U, Puri P.** Postnatal development of the mucosal plexus in the porcine small and large intestine. *Pediatr Surg Int.* 2006; 22: 997–1001.
78. **Young HM, Ciampoli D.** Transient expression of neuronal nitric oxide synthase by neurons of the submucous plexus of the mouse small intestine. *Cell Tissue Res.* 1998; 291: 395–401.
79. **Bredt DS, Snyder SH.** Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia,

and olfactory epithelium. *Neuron.* 1994; 13: 301–13.

80. **Anderson RL, Morris JL, Gibbins IL.** Neurochemical differentiation of functionally distinct populations of autonomic neurons. *J Comp Neurol.* 2001; 429: 419–35.
81. **Baetge G, Pintar JE, Gershon MD.** Transiently catecholaminergic (TC) cells in the bowel of the fetal rat: precursors of noncatecholaminergic enteric neurons. *Dev Biol.* 1990; 141: 353–80.
82. **Baetge G, Schneider KA, Gershon MD.** Development and persistence of catecholaminergic neurons in cultured explants of fetal murine vagus nerves and bowel. *Development.* 1990; 110: 689–701.
83. **Pisano JM, Birren SJ.** Restriction of developmental potential during divergence of the enteric and sympathetic neuronal lineages. *Development.* 1999; 126: 2855–68.
84. **Li ZS, Pham TD, Tamir H, *et al.*** Enteric dopaminergic neurons: definition, developmental lineage, and effects of extrinsic denervation. *J Neurosci.* 2004; 24: 1330–9.
85. **Furness JB, Li ZS, Young HM, *et al.*** Nitric oxide synthase in the enteric nervous system of the guinea-pig: a quantitative description. *Cell Tissue Res.* 1994; 277: 139–49.
86. **Rothman TP, Gershon MD, Holtzer H.** The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursors. *Dev Biol.* 1978; 65: 322–41.
87. **Young HM, Turner KN, Bergner AJ.** The location and phenotype of proliferating neural-crest-derived cells in the developing mouse gut. *Cell Tissue Res.* 2005; 320: 1–9.
88. **Pham TD, Gershon MD, Rothman TP.** Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. *J Comp Neurol.* 1991; 314: 789–98.
89. **Chalazonitis A, Pham TD, Li Z, *et al.*** Bone morphogenetic protein regulation of enteric neuronal phenotypic diversity: relationship to timing of cell cycle exit. *J Comp Neurol.* 2008; 509: 474–92.
90. **Epstein ML, Saffrey MJ, Poulsen KT.** Development and birthdates of vasoactive intestinal peptide immunoreactive neurons in the chick proventriculus. *J Comp Neurol.* 1992; 321: 83–92.
91. **Furness JB, Kunze WA, Bertrand PP, *et al.*** Intrinsic primary afferent neurons of the intestine. *Prog Neurobiol.* 1998; 54: 1–18.
92. **Mao Y, Wang B, Kunze W.** Characterization of myenteric sensory neurons in the mouse small intestine. *J Neurophysiol.* 2006; 96: 998–1010.
93. **Huckfeldt RM, Schubert T, Morgan JL, *et al.*** Transient neurites of retinal horizontal cells exhibit columnar tiling *via* homotypic interactions. *Nat Neurosci.* 2009; 12: 35–43.
94. **Young HM, Furness JB, Povey JM.** Analysis of connections between nitric oxide synthase neurons in the myenteric plexus of the guinea-pig small intestine. *J Neurocytol.* 1995; 24: 257–63.
95. **Vohra BP, Fu M, Heuckeroth RO.** Protein kinase Czeta and glycogen synthase kinase-3beta control neuronal polarity in developing rodent enteric neurons, whereas SMAD specific E3 ubiquitin protein ligase 1 promotes neurite growth but does not influence polarity. *J Neurosci.* 2007; 27: 9458–68.
96. **Sato Y, Heuckeroth RO.** Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth *in vitro*. *Dev Biol.* 2008; 320: 185–98.
97. **Faussone-Pellegrini MS, Matini P, DeFelici M.** The cytoskeleton of the myenteric neurons during murine embryonic life. *Anat Embryol.* 1999; 199: 459–69.
98. **Jiang Y, Liu MT, Gershon MD.** Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas. *Dev Biol.* 2003; 258: 364–84.
99. **Anderson RB, Bergner AJ, Taniguchi M, *et al.*** Effects of different regions of the developing gut on the migration of enteric neural crest-derived cells: a role for Sema3A, but not Sema3F. *Dev Biol.* 2007; 305: 287–99.
100. **Shepherd IT, Raper JA.** Collapsin-1/semaphorin D is a repellent for chick ganglion of Remak axons. *Dev Biol.* 1999; 212: 42–53.
101. **Young HM.** Functional development of the enteric nervous system–from migration to motility. *Neurogastroenterol Motil.* 2008; 20: 20–31.
102. **Bornstein JC, Costa M, Grider JR.** Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil.* 2004; 16: S34–8.
103. **Stewart AL, Young HM, Popoff M, *et al.*** Effects of pharmacological inhibition of small GTPases on axon extension and migration of enteric neural crest-derived cells. *Dev Biol.* 2007; 307: 92–104.
104. **Vannucchi MG, Faussone-Pellegrini MS.** Synapse formation during neuron differentiation: an *in situ* study of the myenteric plexus during murine embryonic life. *J Comp Neurol.* 2000; 425: 369–81.
105. **Vohra BP, Tsuji K, Nagashimada M, *et al.*** Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. *Dev Biol.* 2006; 298: 259–71.
106. **Coventry S, Yost C, Palmiter RD, *et al.*** Migration of ganglion cell precursors in the ileoceca of normal and lethal spotted embryos, a murine model for Hirschsprung disease. *Lab Invest.* 1994; 71: 82–93.
107. **Qian X, Shen Q, Goderie SK, *et al.*** Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron.* 2000; 28: 69–80.
108. **Bates MD, Dunagan DT, Welch LC, *et al.*** The Hlx homeobox transcription factor is required early in enteric nervous system development. *BMC Dev Biol.* 2006; 6: 33.
109. **Blaugrund E, Pham TD, Tennyson VM, *et al.*** Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *Development.* 1996; 122: 309–20.
110. **Okamura Y, Saga Y.** Notch signaling is required for the maintenance of enteric neural crest progenitors. *Development.* 2008; 135: 3555–65.
111. **Kapur RP.** Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. *Pediatr Dev Pathol.* 1999; 2: 559–69.
112. **Paratore C, Goerich DE, Suter U, *et al.*** Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development.* 2001; 128: 3949–61.
113. **Paratore C, Hagedorn L, Floris J, *et al.*** Cell-intrinsic and cell-extrinsic cues regulating lineage decisions in multipotent neural crest-derived progenitor cells. *Int J Dev Biol.* 2002; 46: 193–200.
114. **Southard-Smith EM, Kos L, Pavan WJ.** Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat Genet.* 1998; 18: 60–4.
115. **Young HM, Bergner AJ, Muller T.** Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. *J Comp Neurol.* 2003; 456: 1–11.

116. **D'Autreaux F, Morikawa Y, Cserjesi P, *et al.*** Hand2 is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia. *Development.* 2007; 134: 2237–49.
117. **Hendershot TJ, Liu H, Sarkar AA, *et al.*** Expression of Hand2 is sufficient for neurogenesis and cell type-specific gene expression in the enteric nervous system. *Dev Dyn.* 2007; 236: 93–105.
118. **Pattyn A, Morin X, Cremer H, *et al.*** The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature.* 1999; 399: 366–70.
119. **Lang D, Chen F, Milewski R, *et al.*** Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. *J Clin Invest.* 2000; 106: 963–71.
120. **Heng JI, Nguyen L, Castro DS, *et al.*** Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. *Nature.* 2008; 455: 114–8.
121. **Nobrega-Pereira S, Kessaris N, Du T, *et al.*** Postmitotic Nkx2–1 controls the migration of telencephalic interneurons by direct repression of guidance receptors. *Neuron.* 2008; 59: 733–45.
122. **Young HM, Hearn CJ, Ciampoli D, *et al.*** A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture. *Dev Biol.* 1998; 202: 67–84.
123. **Corpening JC, Cantrell VA, Deal KK, *et al.*** A Histone2BCerulean BAC transgene identifies differential expression of Phox2b in migrating enteric neural crest derivatives and enteric glia. *Dev Dyn.* 2008; 237: 1119–32.
124. **Pattyn A, Simplicio N, van Doorninck JH, *et al.*** Ascl1/Mash1 is required for the development of central serotonergic neurons. *Nat Neurosci.* 2004; 7: 589–95.
125. **Cheng L, Samad OA, Xu Y, *et al.*** Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. *Nat Neurosci.* 2005; 8: 1510–5.
126. **Flames N, Hobert O.** Gene regulatory logic of dopamine neuron differentiation. *Nature.* 2009;
127. **Goridis C, Rohrer H.** Specification of catecholaminergic and serotonergic neurons. *Nat Rev Neurosci.* 2002; 3: 531–41.
128. **Schuchardt A, D'Agati V, Larsson-Blomberg L, *et al.*** Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature.* 1994; 367: 380–3.
129. **Taraviras S, Marcos-Gutierrez CV, Durbec P, *et al.*** Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. *Development.* 1999; 126: 2785–97.
130. **Newgreen D, Young HM.** Enteric nervous system: development and developmental disturbances-part 1. *Pediatr Dev Pathol.* 2002; 5: 224–47.
131. **Newgreen D, Young HM.** Enteric nervous system: development and developmental disturbances–part 2. *Pediatr Dev Pathol.* 2002; 5: 329–49.
132. **Enomoto H.** Regulation of neural development by glial cell line-derived neurotrophic factor family ligands. *Anat Sci Int.* 2005; 80: 42–52.
133. **Heanue TA, Pachnis V.** Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. *Nat Rev Neurosci.* 2007; 8: 466–79.
134. **Heanue TA, Pachnis V.** Ret isoform function and marker gene expression in the enteric nervous system is conserved across diverse vertebrate species. *Mech Dev.* 2008; 125: 687–99.
135. **Durbec PL, Larsson-Blomberg LB, Schuchardt A, *et al.*** Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development.* 1996; 122: 349–58.
136. **Moore MW, Klein RD, Farinas I, *et al.*** Renal and neuronal abnormalities in mice lacking GDNF. *Nature.* 1996; 382: 76–9.
137. **Pichel JG, Shen L, Sheng HZ, *et al.*** GDNF is required for kidney development and enteric innervation. *Cold Spring Harb Symp Quant Biol.* 1996; 61: 445–57.
138. **Sanchez MP, Silos-Santiago I, Frisen J, *et al.*** Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature.* 1996; 382: 70–3.
139. **Golden JP, DeMaro JA, Osborne PA, *et al.*** Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. *Exp Neurol.* 1999; 158: 504–28.
140. **Natarajan D, Marcos-Gutierrez C, Pachnis V, *et al.*** Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. *Development.* 2002; 129: 5151–60.
141. **Pachnis V, Mankoo B, Costantini F.** Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development.* 1993; 119: 1005–17.
142. **Chalazonitis A, Rothman TP, Chen J, *et al.*** Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 *in vitro* and in vivo. *Dev Biol.* 1998; 204: 385–406.
143. **Hearn CJ, Murphy M, Newgreen D.** GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons *in vitro*. *Dev Biol.* 1998; 197: 93–105.
144. **Heuckeroth RO, Lampe PA, Johnson EM, *et al.*** Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. *Dev Biol.* 1998; 200: 116–29.
145. **Natarajan D, Grigoriou M, Marcos-Gutierrez CV, *et al.*** Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture. *Development.* 1999; 126: 157–68.
146. **Young HM, Hearn CJ, Farlie PG, *et al.*** GDNF is a chemoattractant for enteric neural cells. *Dev Biol.* 2001; 229: 503–16.
147. **Gianino S, Grider JR, Cresswell J, *et al.*** GDNF availability determines enteric neuron number by controlling precursor proliferation. *Development.* 2003; 130: 2187–98.
148. **Roberts RR, Bornstein JC, Bergner AJ, *et al.*** Disturbances of colonic motility in mouse models of Hirschsprung's disease. *Am J Physiol Gastrointest Liver Physiol.* 2008; 294: G996-G1008.
149. **Rossi J, Luukko K, Poteryaev D, *et al.*** Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor. *Neuron.* 1999; 22: 243–52.
150. **Barlow A, de Graaff E, Pachnis V.** Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. *Neuron.* 2003; 40: 905–16.
151. **Leibl MA, Ota T, Woodward MN, *et al.*** Expression of endothelin 3 by mesenchymal cells of embryonic mouse caecum. *Gut.* 1999; 44: 246–52.
152. **Lee HO, Levorse JM, Shin MK.** The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. *Dev Biol.* 2003; 259: 162–75.
153. **Sidebotham EL, Woodward MN, Kenny SE, *et al.*** Localization and endothelin-3

dependence of stem cells of the enteric nervous system in the embryonic colon. *J Pediatr Surg.* 2002; 37: 145–50.

154. **Baynash AG, Hosoda K, Giaid A, *et al.*** Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell.* 1994; 79: 1277–85.
155. **Hosoda K, Hammer RE, Richardson JA, *et al.*** Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell.* 1994; 79: 1267–76.
156. **Wu JJ, Chen JX, Rothman TP, *et al.*** Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. *Development.* 1999; 126: 1161–73.
157. **Nagy N, Goldstein AM.** Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. *Dev Biol.* 2006; 293: 203–17.
158. **Bondurand N, Natarajan D, Barlow A, *et al.*** Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling. *Development.* 2006; 133: 2075–86.
159. **Woodward MN, Sidebotham EL, Connell MG, *et al.*** Analysis of the effects of endothelin-3 on the development of neural crest cells in the embryonic mouse gut. *J Pediatr Surg.* 2003; 38: 1322–8.
160. **Sandgren K, Larsson LT, Ekblad E.** Widespread changes in neurotransmitter expression and number of enteric neurons and interstitial cells of Cajal in lethal spotted mice: an explanation for persisting dysmotility after operation for Hirschsprung's disease? *Dig Dis Sci.* 2002; 47: 1049–64.
161. **Chalazonitis A.** Neurotrophin-3 in the development of the enteric nervous system. *Prog Brain Res.* 2004; 146: 243–63.
162. **Chalazonitis A, Pham TD, Rothman TP, *et al.*** Neurotrophin-3 is required for the survival-differentiation of subsets of developing enteric neurons. *J Neurosci.* 2001; 21: 5620–36.
163. **Pisano JM, Colon-Hastings F, Birren SJ.** Postmigratory enteric and sympathetic neural precursors share common, developmentally regulated, responses to BMP2. *Dev Biol.* 2000; 227: 1–11.
164. **Goldstein AM, Brewer KC, Doyle AM, *et al.*** BMP signaling is necessary for neural crest cell migration and ganglion formation in the enteric nervous system. *Mech Dev.* 2005; 122: 821–33.
165. **Fu M, Vohra BP, Wind D, *et al.*** BMP signaling regulates murine enteric nervous system precursor migration, neurite fasciculation, and patterning via altered Ncam1 polysialic acid addition. *Dev Biol.* 2006; 299: 137–50.
166. **Faure C, Chalazonitis A, Rheaume C, *et al.*** Gangliogenesis in the enteric nervous system: roles of the polysialylation of the neural cell adhesion molecule and its regulation by bone morphogenetic protein-4. *Dev Dyn.* 2007; 236: 44–59.
167. **Chalazonitis A, D'Autreaux F, Guha U, *et al.*** Bone morphogenetic protein-2 and -4 limit the number of enteric neurons but promote development of a TrkC-expressing neurotrophin-3-dependent subset. *J Neurosci.* 2004; 24: 4266–82.
168. **Anderson RB, Turner KN, Nikonenko AG, *et al.*** The cell adhesion molecule L1 is required for chain migration of neural crest cells in the developing mouse gut. *Gastroenterology.* 2006; 130: 1221–32.
169. **Turner KN, Schachner M, Anderson RB.** Cell adhesion molecule L1 affects the rate of differentiation of enteric neurons in the developing gut. *Dev Dyn.* 2009; 238: 708–15.
170. **Fu M, Lui VC, Sham MH, *et al.*** Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. *J Cell Biol.* 2004; 166: 673–84.
171. **Ramalho-Santos M, Melton DA, McMahon AP.** Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development.* 2000; 127: 2763–72.
172. **Reichenbach B, Delalande JM, Kolmogorova E, *et al.*** Endoderm-derived Sonic hedgehog and mesoderm Hand2 expression are required for enteric nervous system development in zebrafish. *Dev Biol.* 2008; 318: 52–64.
173. **Root CM, Velazquez-Ulloa NA, Monsalve GC, *et al.*** Embryonically expressed GABA and glutamate drive electrical activity regulating neurotransmitter specification. *J Neurosci.* 2008; 28: 4777–84.
174. **Chevalier J, Derkinderen P, Gomes P, *et al.*** Activity-dependent regulation of tyrosine hydroxylase expression in the enteric nervous system. *J Physiol.* 2008; 586: 1963–75.
175. **Ruhl A.** Glial cells in the gut. *Neurogastroenterol Motil.* 2005; 17: 777–90.
176. **Bush TG, Savidge TC, Freeman TC, *et al.*** Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell.* 1998; 93: 189–201.
177. **Aube AC, Cabarrocas J, Bauer J, *et al.*** Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. *Gut.* 2006; 55: 630–7.
178. **Gomes P, Chevalier J, Boesmans W, *et al.*** ATP-dependent paracrine communication between enteric neurons and glia in a primary cell culture derived from embryonic mice. *Neurogastroenterol Motil.* 2009;
179. **Gulbransen BD, Sharkey KA.** Purinergic neuron-to-glia signaling in the enteric nervous system. *Gastroenterology.* 2009; 136: 1349–58.
180. **Bassotti G, Villanacci V, Antonelli E, *et al.*** Enteric glial cells: new players in gastrointestinal motility? *Lab Invest.* 2007; 87: 628–32.
181. **Rothman TP, Tennyson VM, Gershon MD.** Colonization of the bowel by the precursors of enteric glia: studies of normal and congenitally aganglionic mutant mice. *J Comp Neurol.* 1986; 252: 493–506.
182. **Britsch S, Goerich DE, Riethmacher D, *et al.*** The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* 2001; 15: 66–78.
183. **Morrison SJ, Perez SE, Qiao Z, *et al.*** Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell.* 2000; 101: 499–510.
184. **Wakamatsu Y, Maynard TM, Weston JA.** Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development.* 2000; 127: 2811–21.
185. **Taylor MK, Yeager K, Morrison SJ.** Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. *Development.* 2007; 134: 2435–47.
186. **Fu M, Tam PK, Sham MH, *et al.*** Embryonic development of the ganglion plexuses and the concentric layer structure of human gut: a topographical study. *Anat Embryol.* 2004; 208: 33–41.
187. **Wallace AS, Burns AJ.** Development of the enteric nervous system, smooth muscle and interstitial cells of Cajal in the human gastrointestinal tract. *Cell Tissue Res.* 2005; 319: 367–82.
188. **McKeown SJ, Chow CW, Young HM.** Development of the submucous plexus in the large intestine of the mouse. *Cell Tissue Res.* 2001; 303: 301–5.
189. **Tam PK.** An immunochemical study with neuron-specific-enolase and substance P of human enteric innervation–the normal developmental pattern and abnormal deviations in Hirschsprung's disease and pyloric stenosis. *J Pediatr Surg.* 1986; 21: 227–32.

190. **Fekete E, Timmermans JP, Resch BA, *et al.*** Different distribution of S-100 protein and glial fibrillary acidic protein (GFAP) immunoreactive cells and their relations with nitrergic neurons in the human fetal small intestine. *Histol Histopathol.* 1999; 14: 785–90.
191. **Timmermans JP, Barbiers M, Scheuermann DW, *et al.*** Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. *Cell Tissue Res.* 1994; 275: 235–45.
192. **Brandt CT, Tam PK, Gould SJ.** Nitrergic innervation of the human gut during early fetal development. *J Pediatr Surg.* 1996; 31: 661–4.
193. **Rauch U, Klotz M, Maas-Omlor S, *et al.*** Expression of intermediate filament proteins and neuronal markers in the human fetal gut. *J Histochem Cytochem.* 2006; 54: 39–46.
194. **White FV, Langer JC.** Circumferential distribution of ganglion cells in the transition zone of children with Hirschsprung disease. *Pediatr Dev Pathol.* 2000; 3: 216–22.
195. **Barshack I, Fridman E, Goldberg I, *et al.*** The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. *J Clin Pathol.* 2004; 57: 712–6.
196. **Kapur RP, Reed RC, Finn LS, *et al.*** Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung Disease. *Pediatr Dev Pathol.* 2009; 12: 6–15.
197. **Bettolli M, De Carli C, Jolin-Dahel K, *et al.*** Colonic dysmotility in postsurgical patients with Hirschsprung's disease. Potential significance of abnormalities in the interstitial cells of Cajal and the enteric nervous system. *J Pediatr Surg.* 2008; 43: 1433–8.
198. **Taguchi T, Suita S, Masumoto K, *et al.*** An abnormal distribution of C-kit positive cells in the normoganglionic segment can predict a poor clinical outcome in patients with Hirschsprung's disease. *Eur J Pediatr Surg.* 2005; 15: 153–8.
199. **De Giorgio R, Guerrini S, Barbara G, *et al.*** New insights into human enteric neuropathies. *Neurogastroenterol Motil.* 2004; 16: S143–7.
200. **Gariepy CE.** Developmental disorders of the enteric nervous system: genetic and molecular bases. *J Pediatr Gastroenterol Nutr.* 2004; 39: 5–11.
201. **Chitkara DK, Di Lorenzo C.** From the bench to the 'crib'-side: implications of scientific advances to paediatric neurogastroenterology and motility. *Neurogastroenterol Motil.* 2006; 18: 251–62.
202. **Smith VV.** Intestinal neuronal density in childhood: a baseline for the objective assessment of hypo- and hyperganglionosis. *Pediatr Pathol.* 1993; 13: 225–37.
203. **Vanderwinden JM, Mailleux P, Schiffmann SN, *et al.*** Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. *N Engl J Med.* 1992; 327: 511–5.
204. **Hutson JM, Chow CW, Borg J.** Intractable constipation with a decrease in substance P-immunoreactive fibres: is it a variant of intestinal neuronal dysplasia? *J Pediatr Surg.* 1996; 31: 580–3.
205. **Stanton MP, Hengel PT, Southwell BR, *et al.*** Cholinergic transmission to colonic circular muscle of children with slow-transit constipation is unimpaired, but transmission via NK2 receptors is lacking. *Neurogastroenterol Motil.* 2003; 15: 669–78.
206. **Taketomi T, Yoshiga D, Taniguchi K, *et al.*** Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and esophageal achalasia. *Nat Neurosci.* 2005; 8: 855–7.
207. **Eng C, Mulligan LM.** Mutations of the RET proto-oncogene in the multiple endocrine neoplasia type 2 syndromes, related sporadic tumours, and hirschsprung disease. *Hum Mutat.* 1997; 9: 97–109.
208. **Smith VV, Eng C, Milla PJ.** Intestinal ganglioneuromatosis and multiple endocrine neoplasia type 2B: implications for treatment. *Gut.* 1999; 45: 143–6.
209. **King SK, Southwell BR, Hutson JM.** An association of multiple endocrine neoplasia 2B, a RET mutation; constipation; and low substance P-nerve fiber density in colonic circular muscle. *J Pediatr Surg.* 2006; 41: 437–42.
210. **Yin M, King SK, Hutson JM, *et al.*** Multiple endocrine neoplasia type 2B diagnosed on suction rectal biopsy in infancy: a report of 2 cases. *Pediatr Dev Pathol.* 2006; 9: 56–60.
211. **Kapur RP.** Neuronal dysplasia: a controversial pathological correlate of intestinal pseudo-obstruction. *Am J Med Genet.* 2003; 122A: 287–93.
212. **Meier-Ruge WA, Bruder E, Kapur RP.** Intestinal neuronal dysplasia type B: one giant ganglion is not good enough. *Pediatr Dev Pathol.* 2006; 9: 444–52.
213. **Knowles CH, De Giorgio R, Kapur RP, *et al.*** Gastrointestinal neuromuscular pathology: guidelines for histological techniques and reporting on behalf of the Gastro 2009 International Working Group. *Acta Neuropathol.* 2009; 118: 271–301.
214. **Meier-Ruge WA, Ammann K, Bruder E, *et al.*** Updated results on intestinal neuronal dysplasia (IND B). *Eur J Pediatr Surg.* 2004; 14: 384–91.
215. **Cord-Udy CL, Smith VV, Ahmed S, *et al.*** An evaluation of the role of suction rectal biopsy in the diagnosis of intestinal neuronal dysplasia. *J Pediatr Gastroenterol Nutr.* 1997; 24: 1–6.
216. **Koletzko S, Jesch I, Faus-Kebetaler T, *et al.*** Rectal biopsy for diagnosis of intestinal neuronal dysplasia in children: a prospective multicentre study on interobserver variation and clinical outcome. *Gut.* 1999; 44: 853–61.
217. **Burns AJ.** Migration of neural crest-derived enteric nervous system precursor cells to and within the gastrointestinal tract. *Int J Dev Biol.* 2005; 49: 143–50.
218. **Amiel J, Sproat-Emison E, Garcia-Barcelo M, *et al.*** Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet.* 2008; 45: 1–14.
219. **Enomoto H, Crawford PA, Gorodinsky A, *et al.*** RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. *Development.* 2001; 128: 3963–74.