Development of the Enteric Nervous System

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2.1 Introduction

The enteric nervous system (ENS) is the largest and the most complex division of the peripheral nervous system [1]. The ENS contains more neurons than the spinal cord and is capable of mediating reflex activity in the absence of central nervous system. About 80–100 million enteric neurons can be classified into functional distinct subpopulations, including intrinsic primary neurons, interneurons, motor neurons, secretomotor and vasomotor neurons [2]. The ENS plays a crucial role in normal gastrointestinal motility. Therefore insights into the development of the gastrointestinal tract and the ENS are relevant for the understanding of the pathophysiology and treatment of infants and children with motility disorders.

2.2 Embryonic Origin of ENS

There are two major steps in the development of the gastrointestinal tract: (1) formation of the gut tube, and (2) formation of individual organs, each with their specialized cell types (Table 2.1) [3].

Gastrulation is an early step in the development of all multicellular organisms. During gastrulation the axes of the embryo are determined and the development of the gastrointestinal tract starts. Gastrulation gives rise to three germ layers, endoderm, mesoderm, and ectoderm [3]. The mammalian gastrointestinal system originates from all three embryonic germ layers. The epithelial lining of the gastrointestinal tube and the parenchymal cells of the liver and pancreas are formed by the endoderm. The mesoderm provides mesenchymal elements including smooth muscle and stromal cells. The neurons of the ENS which regulates gastrointestinal motility are derived from ectoderm.

The ectoderm divides into three types of cells; outer ectoderm, neural tube, and neural crest (NC). The NC arises from the dorsal region of the neural tube. Melanocytes, the adrenal medulla, the dentine of teeth, the sympathetic and parasympathetic arms of the peripheral nervous system, and the neurons of the ENS are derived form the NC. These tissues and cell types originate from

Table 2.1 Developmental milestones of human gastrointestinal tract

Developmental stage	Gestation week
Gastrulation	3
Gut tube largely closed	4
Liver and pancreas buds	4
Growth of intestines into cord	7
Intestinal villus formation	8
Retraction of intestines into abdominal cavity	10
Organ formation complete	12
Parietal cells detectable, pancreatic islets appear, bile secretion, intestinal enzymes detectable	12
Swallowing detectable	16 and 17
Mature motility	36

different regions of the NC, which means that the cells need to migrate to the site of the mature organs. The gene mutations that result in disrupted NC cell migration to one region also cause altered migration of other NC-derived tissues [4].

2.3 Origin and Development of Neural Crest-Derived Cells

The NC is located along the entire length of the body axis. Two groups of undifferentiated cells, derived from NCs, colonize the gut wall and migrate in craniocaudal and caudocranial directions.

The embryonic NC arises in the neural tube, originating with the central nervous system, but NC cells detach from this tissue via reduction of cell–cell and cell–matrix adhesion. The epitheliomesenchymal transformation allows NC cells to migrate along pathways of defined routes to various tissues, where they stop moving and differentiate into various cell types. Pathway selection is most likely achieved by balanced combinations of molecules that promote and reduce adhesions [5, 6]. NC cells give rise to neuronal, endocrine and paraendocrine, craniofacial, conotruncal heart, and pigmentary tissues. Neurocristopathies encompass tumors, malformations, and single or multiple abnormalities of tissues, mentioned above in various combinations [7].

In the human fetus, NC-derived cells first appear in the developing esophagus at the 5th week of gestation, and then migrate down to the anal canal in a craniocaudal direction during the 5th and 12th week of gestation. The NC cells first form the myenteric plexus just outside the circular muscle layer. The mesenchymally derived longitudinal muscle layer then forms, sandwiching the myenteric plexus after it has been formed in the 12th week of gestation. In addition, after the craniocaudal migration has ended, the submucous plexus is formed by the neuroblasts, which migrate from the myenteric plexus across the circular muscle layer and into the submucosa; this progresses in a craniocaudal direction during the 12th to 16th week of gestation [5]. The absence of ganglion cells in Hirschsprung's disease has been attributed to a failure of migration of NC cells. The earlier the arrest of migration, the longer the aganglionic segment is.

It is generally accepted that the enteric ganglion cells are derived primarily from the NC cells [8–11]. Studies in the avian system provide strong evidence for the contribution of the sacral NC to the hindgut ENS [12–14]. Whether the sacral NC contributes to the ENS in the mammalian hindgut is less clear. Failure of the vagal derived NC cells to colonize the hindgut results in failure of hindgut ENS development, suggesting that interaction between sacral and vagal enteric NC cells may be necessary for sacral NC cell contribution to the ENS [15].

Yntma and Hammond first performed NC ablations in chick embryos and identified the vagal NC (somites 1 to 7) as the source of the ENS stem cells [11]. Le Douarin and Teillet showed an additional source of NC stem cells originating from the lumbosacral region to colonize the gut [12]. Later the lumbosacral derived crest cells were found principally in the myenteric plexus, with very few in the submucous plexus. The number of these cells declines rostrally. Cells derived from the lumbosacral NC were never observed in any gut region above the umbilicus [14].

The colonization of the gut by sacral NC-derived cells and the contribution of the cells to the development of the ENS is controversial [16]. The dual origin of enteric neurons has been negated by studies on chick embryo as well as human embryo. Allen and Newgreen [17] isolated bowel segments from fowl embryos at various stages of development, and grew these segments in the chorioallantoic membrane and found that enteric neurons appeared in a craniocaudal sequence, showing a vagal source. Meijers et al. [18] transected the chicken bowel in ovo at an early stage, before the passage of NC cells had occurred, preventing craniocaudal migration of vagal NC cells. They found that the hindgut remained aganglionic, showing that there was no colonization by sacral NC cells.

Some studies have shown that sacral NC-derived cells migrate from the neural plate early in development and extraenteric pelvic ganglia. Later these cells are able to colonize the gut and contribute to the ENS, coincident with the migration of vagal NC-derived cells [14, 19–21]. In contrast, other studies suggest that sacral NC-derived cells invade the hindgut mesenchyme several days before the colonization of the hindgut by vagal NC cells and contribute to the development of ENS [13, 22–24].

In contrast the mouse ENS is derived embryologically from cells of the vagal, truncal, and sacral regions of the NC. The vagal NC originates in somites 1 to 5 in the mouse, the truncal NC from somites 6 and 7, and the sacral NC posterior to somite 28. Cells from each of these regions of the NC migrate into the developing gut by defined pathways. Cells of the vagal and truncal NC enter the foregut, migrating in a proximal to distal direction. Truncal NC cells populate only the foregut, whereas those of the vagal NC migrate more distally to colonize the rest of the gastrointestinal tract. Cells arising from the sacral crest seem first to colonize pelvic autonomic ganglia, from which they then migrate into the distal gut, colonizing it from distal to proximal [19].

The current concept is that the development of the ENS in humans is derived primarily from cells of the vagal segment of the NC [2, 12]. Fujimoto et al. [25] studied NC cell migration in the developing gut in the human embryo using antineurofilament protein triplet antibody and found that enteric ganglia originated from a single vagal NC source. The vast majority of studies have revealed that vagal NC cells provide the main source of enteric neurons and sacral NC additionally innervates the distal bowel [12–14, 26–28].

The final requirement for development and maturation of the ENS is the formation of ganglia. Several days after NC cells have colonized the gut these cells are evenly distributed, with no indication of cell clustering, except the cecum. As the gut later increases in length and diameter, the cells start forming ganglionic groups [29]. A previous study has shown that cells forming a ganglion do not arise from a single precursor cell [30]. A recent study used human fetal intestine to investigate nitrergic neurons in the developing myenteric plexus. The distribution of nitrergic neurons was found to change markedly between 14 and 22 weeks of gestation. Nitrergic neurons were randomly distributed at week 14 and were later aggregated in the plexus and within individual ganglia at week 19 [31]. It is currently not known what factors induce cells to cluster into ganglia.

2.4 Functional Development of the ENS

The complexity of mature ENS is exemplified by many different functional types of neurons containing various neurotransmitters occurring in various combinations. Types of neurotransmitters vary according to the time of their appearance [29, 32]. The development of the human enteric nervous system is characterized by the early appearance (between 9 and 12 weeks' gestation) of adrenergic and cholinergic nerves. Strong evidence has emerged that the enteric nervous system is not only composed of adrenergic and cholinergic nerves but also nonadrenergic, noncholinergic (NANC) autonomic nerves, which contain different peptides. These peptides act as neurotransmitters, or neuromodulators, or both. These nerves have been termed *peptidergic nerves*. The development of peptidergic innervation occurs much later.

In recent years, pharmacologic and physiologic studies have provided evidence that nitric oxide (NO) is the most important mediator in nonadrenergic, noncholinergic relaxation of the gastrointestinal tract. By 12 weeks' gestation, nitrergic neurons appear in the myenteric ganglia, at all levels of the gut, and begin plexus formation. Nitrergic innervation in the submucous plexus becomes evident after 14 weeks. As gestational age increases, nitrergic innervation becomes richer and more organized. Increasing numbers of nitrergic nerve fibers are seen in the circular muscle; some of these fibers project from the myenteric plexus. Thus, the onset and pace of development of nitrergic innervation are similar to adrenergic and cholinergic innervation and occur before peptidergic innervation [33].

Serotonin (5-HT) together with glucagon, insulin, peptide XY, gastrin, and somatostatin are the earliest neurohumoral substances to be expressed at about 8 weeks of gestation. By 24 weeks of gestation, most of the known gastrointestinal neurohumoral substances can be identified.

Further contacts between enteric nerves and effectors are developed at 26 weeks and the first signs of motility can be detected at 25 weeks of gestation [3].

2.5 Development of Intestinal Motility

The innervation of the gastrointestinal tract in utero is accompanied by functional activity of increasing complexity. The first studies to measure intestinal transit in humans used amniography; aboral transport of contrast agent did not occur in the intestinal tract of fetuses younger than 30 weeks of gestation [34]. With increasing gestational age, increasing aboral transit and rate of propagation develops. Subsequent studies of gastrointestinal motility in premature infants have been performed using intraluminal catheters [35]. The data from these studies reveal no regular periodicity or rhythmicity at 25 weeks of gestation. Further development occurs during the next 15 weeks, so that by term, mature motor patterns of the gastrointestinal tract are well established. Responses to feeding vary considerably among preterm infants; in general, intestinal motility studies can predict feeding intolerance [36].

Enteric nerve cells continue to differentiate throughout the first couple of years of life, which means that the infant's nervous system is plastic and developing [37]. There is clear evidence that the development of the ENS continues after birth. In rats, NO synthase-expressing neurons are already present at birth but increase in number and location during the first 3 weeks of postnatal life [32]. Normal ganglion cell distribution is present at 24 weeks of gestation in humans. These ganglia continue to mature on into childhood. Previous studies on human bowel specimens have revealed that the density of NADPH-diaphorase-positive ganglion cells decreases in the submucous plexus of the human distal colon and the myenteric plexus of human small bowel, colon and rectum [38, 39].

2.6 Genes Involved in ENS Development

Normal development of ENS is related to migration, proliferation, differentiation and survival of NC-derived cells [40]. Several genes and signaling molecules have been identified that control morphogenesis and differentiation of the ENS. These genes, when mutated or deleted, interfere with ENS development (Table 2.2) [7, 42–44].

2.6.1 RET/GDNF/GFRα1 Signaling System

This signaling pathway is of importance for subpopulations of both peripheral and central neurons, having been shown by in vitro and in vivo assays to promote survival of neurons, mitosis of neuronal progenitor cells, and dif-

Table 2.2 Genes involved in the morphogenesis and differentiation of the ENS

Genes	Chromosomal assignment	Function
RET	10q11.2	Tyrosine kinase receptor
GDNF	5p12–13.1	Glial cell-derived neurotropic factor
NTN	19q13.3	Neurturin, RET ligand
GFRα	10q26	GDNF family receptor alpha 1
EDNRB	13q22	Endothelin-B receptor
EDN-3	20q13.2–13.3	Endothelin-B
ECE-1	1p36.1	Endothelin-converting enzyme
SOX 10	22q13.1	Sry/HMG box transcription factor
PHOX2B	4p12	Paired-like homeobox 2b
PAX3	2q35	Paired box gene 3
SIP1	2q22	Siah-interacting protein

ferentiation of neurons and neurite extension [41, 45, 46]. The RET receptor is the signaling component of receptor complexes with four ligands, glial derived neurotropic factor (GDNF), neurturin (NTN), artemin (ATM), and persephin (PSP) [45, 47]. The complete receptor complex includes the RET receptor tyrosine kinase and a glycosylphosphatidylinositol-anchored binding component (GFRα1, GFRα2, GFRα3, and GFRα4) [47–49]. In vivo the absence of GDNF/GFRα1-mediated signaling leads to the failure of ENS development, whereas the absence of NTN/GFRα2-mediated signaling leads to more subtle abnormalities in ENS development [47]. The importance of RET in mammalian organogenesis has been further illustrated by the generation of RET knockout mice [50]. These mice exhibit total intestinal aganglionosis and renal agenesis. The RET protooncogene has been demonstrated to be a major gene causing Hirschsprung's disease [51–55]. Mutations of RET account for 50% of familial and 15% to 20% of sporadic cases of Hirschsprung's disease [55, 56].

The development of the ENS is dependent upon the actions of GDNF, which stimulates the proliferation and survival of NC-derived precursor cells in the embryonic gut [57–60]. It has been reported that GDNF is the ligand of RET [61]. Mice carrying the homozygous null mutation in GDNF have been generated, and these mice demonstrate the lack of kidneys and ENS, confirming the crucial role of GDNF in the development of the ENS [62, 63]. Although a causative role for GDNF mutations in some patients with Hirschsprung's disease has been suggested, the occurrence of such cases is uncommon, and it is more likely that the GDNF mutations are involved in modulation of the Hirschsprung's disease phenotype via its interaction with other susceptibility loci such as RET [7, 64].

2.6.2 Endothelin Signaling Pathway

The endothelins (EDN1, EDN2, and EDN3) are intercellular local messengers that act via the cell surface receptors, EDNRA and EDNRB [45]. EDN is initially produced as an inactive preproendothelin that undergoes two proteolytic steps to produce an active peptide. The first cleavage produces inactive big endothelins, and these are finally cleaved by a specific protease, endothelin-converting enzyme (ECE) to produce biologically active EDN [7, 16, 45].

EDN3 and EDNRB have a role in the migration and development of the ENS [65–67]. In mice in which the EDN3 or EDNRB gene is disrupted, intestinal aganglionosis has been demonstrated experimentally. Several reports suggest that the downregulation of EDN3 expression may play a role in the pathogenesis of Hirschsprung's disease in the sporadic cases [68–74].

ECE1 knockout mice show craniofacial and cardiac abnormalities in addition to colonic aganglionosis [75].

2.6.3 SOX10

The SOX10 (sex determining region Y-box) gene is expressed in neuronal crest derivates that contribute to the formation of the peripheral nervous system during embryogenesis [76, 77]. The involvement of SOX10 in the development of enteric neurons was demonstrated in the Dom (dominant megacolon) mouse model of Hirschsprung's disease which exhibits distal intestinal aganglionosis [76]. Mutations in SOX10 have been identified as a cause of the dominant megacolon mouse and Waardenburg-Shah syndrome in humans, both of which include defects in the ENS and pigmentation abnormalities [78, 79].

2.6.4 PHOX2B

The PHOX2B gene is a homeodomain-containing transcription factor that is involved in neurogenesis and regulates RET expression in mice, in which disruption of the PHOX2B gene results in a Hirschsprung's disease-like phenotype [80, 81]. Enteric PHOX2B expression begins in vagal and truncal NC-derived cells as they invade the foregut mesenchyme and is contained in the adult submucosal and myenteric plexus [81].

2.6.5 HOX11L1

HOX11L1 is a homeobox gene involved in peripheral nervous system development and is reported to play a role in the proliferation or differentiation of NC cell lines. Two different HOX11L1 knockout mouse models have been generated [82, 83]. In both cases, homozygous

mutant mice were viable but developed megacolon at the age of 3 to 5 weeks. Histologic and immunohistochemical analysis showed hyperplasia of myenteric ganglia, a phenotype similar to that observed in human intestinal neuronal dysplasia.

2.7 Other Factors Implicated in the Control of ENS Development

Kit, another receptor with tyrosine kinase activity, is involved in the development of the interstitial cells of Cajal (ICCs) [84]. These are nonneuronal cells that serve as pacemaker cells and are responsible fro the spontaneous, rhythmic, electrical excitatory activity of gastrointestinal smooth muscle. Recent studies have found that the c-kit receptor is essential for the development of the ICCs. Mesenchymal ICC precursors that carry the c-kit receptor require the kit ligand (KL), which can be provided by neuronal cells or smooth muscle cells. According to the influence of the KL from either neuronal or smooth muscle cells, the ICCs develop as either myenteric ICCs or muscular ICCs [85]. These cells are also important in modulating communications between nerve and muscle. Mice with mutations in the KIT gene lack ICCs and have changes in skin pigment and abnormal intestinal motility [86]. No such mutations have been reported in humans so far, but several studies have shown disturbed expression of ICCs in patients with motility disorders [87–91]

Further studies have indicated the importance of the gut microenvironment during development of ENS. Mice lacking EDN-3 show increased expression of laminin, one of extracellular matrix (ECM) proteins, which leads to the conclusion that EDN-3 also affects the environment through which the NC cells migrate [92]. Altered ECM proteins such as tenascin, fibronectin and nidogen have been shown in patients with Hirschsprung's disease which suggests the importance of ECM molecules during development of ENS [93, 94].

2.8 Conclusions

During the past decade there has been an explosion of information about genes that control the development of NC. Molecular-genetic analysis has identified several genes that have a role in the development of Hirschsprung's disease. The major susceptibility gene is RET, which is also involved in multiple endocrine neoplasia type 2. Recently, genetic studies have provided strong evidence in animal models that intestinal neuronal dysplasia (IND) is a real entity. HOX11L1 knockout mice and endothelin B receptor-deficient rats demonstrated abnormalities of the ENS resembling IND type B in humans. These findings support the concept that IND may be linked to a genetic defect [95]. The development of the ENS requires the complex interaction of genes encoding transcription factors, signaling molecules, and their receptors. Normal ENS development is based on survival of NC-derived cells and their coordinated proliferation, movement and differentiation into neurons and glia. These processes are influenced by the microenvironment of the developing gut. Alterations in gene function, defects in NC cells or changes in the gut microenvironment may result in abnormal development of the ENS.

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