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Mouse models of Hirschsprung disease and other developmental disorders of the enteric nervous system: Old and new players

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ABSTRACT

Hirschsprung disease (HSCR, intestinal aganglionosis) is a multigenic disorder with variable penetrance and severity that has a general population incidence of 1/5000 live births. Studies using animal models have contributed to our understanding of the developmental origins of HSCR and the genetic complexity of this disease. This review summarizes recent progress in understanding control of enteric nervous system (ENS) development through analyses in mouse models. An overview of signaling pathways that have long been known to control the migration, proliferation and differentiation of enteric neural progenitors into and along the developing gut is provided as a framework for the latest information on factors that influence enteric ganglia formation and maintenance. Newly identified genes and additional factors beyond discrete genes that contribute to ENS pathology including regulatory sequences, miRNAs and environmental factors are also introduced. Finally, because HSCR has become a paradigm for complex oligogenic diseases with non-Mendelian inheritance, the importance of gene interactions, modifier genes, and initial studies on genetic background effects are outlined.

1. Introduction

Identifying the factors that lead to enteric ganglia deficiencies, such as Hirschsprung disease (HSCR), has been a long-term goal of many investigators who study innervation of the intestine. Efforts have been particularly focused on genetic factors and the molecular effects of discrete coding and non-coding mutations as well as post-translational mechanisms that impact the enteric neural crest cells (ENCCs) that populate the fetal intestine to form the mature enteric nervous system (ENS). Current knowledge has greatly benefited from exchanges between human geneticists and researchers using animal models. Because gene targeting techniques and inbred strains have long been available in laboratory mice, advances in identifying genes that contribute to HSCR susceptibility and subsequent studies of cellular mechanism have been possible through analysis of ENS development. In recent years, the impact of environmental factors on ENS development has been identified and has been advanced by studies in mouse models (Fu et al., 2010; Heuckeroth and Schafer, 2016; Schill et al., 2016).

The ENS is an extensive network of neurons and glial cells within the wall of the bowel that controls gut motility, regulates transport of ions across the epithelium, and modulates blood flow (Furness, 2012). In the small and large intestine, neurons and glial cells are mostly found in two main plexuses: the myenteric plexus located between the circular and longitudinal muscle layers, and the submucosal plexus found within the connective tissue of the submucosa. The essential role of enteric neurons in peristalsis control is exemplified by bowel obstruction that occurs in aganglionic regions of patients presenting with HSCR (Chakravarti et al., 2004). This multigenic disorder exhibits variable penetrance and severity with a general population incidence of 1/5000 live births and a prominent gender bias of 4:1 in males compared to females (Badner et al., 1990; Spouge and Baird, 1985). Studies using animal models have contributed to our understanding of HSCR genetic complexity through genome targeting efforts in mice that have identified many causative genes for aganglionosis. Continued studies in mice with complementary work in chick and zebrafish, have identified many other molecules that are crucial for ENS development and have aided in understanding cellular processes that occur in normal and abnormal ENS development (see examples included in recent reviews (Goldstein et al., 2013; Harrison and Shepherd, 2013; Lake and Heuckeroth, 2013; Obermayr et al., 2013; Zimmer and Puri, 2015). As a result, we now know that that neural crest cells, mainly from vagal levels of the neural tube,

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enter the foregut and migrate to colonize the whole length of the intestine (Burns and Douarin, 1998; Le Douarin and Teillet, 1974; Young et al., 1998). The principal pathway from vagal levels occurs in a rostro-caudal wave down the length of the developing intestine. Upon reaching the hindgut, ENCCs can proceed either through a trans-mesenteric pathway (Nishiyama et al., 2012) or migrate through the cecum to populate the distal colon (Druckenbrod and Epstein, 2005). Because the gut lengthens substantially while it is being colonized, vagal ENCCs migrate further than any other neural crest cell population. Vagal progenitors are complemented by truncal and sacral ENCC populations that make smaller contributions to total cell numbers (Burns and Douarin, 1998; Kapur, 2000; Wang et al., 2011). In humans, it takes three weeks for these cell populations to colonize the whole length of the bowel, while in mice it takes five days, which is ¼ of the gestation period (for recent review see McKeown et al., 2013; Obermayr et al., 2013). During the whole process, coordinated proliferation, migration, and differentiation is required, as perturbations to ENCCs number, migratory behavior or rate of differentiation can result in aganglionosis of the distal bowel. Although apoptosis is not prominent while ENCCs are colonizing the gut (Gianino et al., 2003), early cell death before vagal crest cells enter the gut has been reported in the chick (Wallace et al., 2009) and is also known to occur among mutants that later exhibit intestinal aganglionosis (Durbec et al., 1996; Kapur, 1999; Stanchina et al., 2006).

This review summarizes recent progress in understanding control of ENS development through analysis of mouse models. An overview of signaling pathways that have long been known to control the migration, proliferation and differentiation of ENCCs into and along the developing gut is provided as a framework for the latest information on factors that influence enteric ganglia formation and maintenance. Newly discovered genes that cause HSCR or other abnormalities of enteric ganglia density such as hypoganglionosis or hyperganglionosis are described. Beyond discrete gene identification, the role of regulatory sequences, miRNAs and environmental factors in the etiology of ENS disorders are also introduced. Finally because HSCR has become a paradigm for complex oligogenic diseases with non-Mendelian inheritance, the importance of gene interactions, modifier genes, and initial studies on genetic background effects are included.

2. Molecular mediators that control ENS development and maturation: Key historical genes and new players

Over the years, multiple naturally occurring (“spontaneous”) or gene-targeted mutations that alter molecules involved in the colonization of the gut by ENCCs have been described. These include factors secreted by the gut mesenchyme that act on receptors expressed by ENCCs, transcription factors, guidance factors and morphogens, as well as proteins that transmit signals from the cell surface to the cytoskeleton and the nucleus, including adhesion molecules. Mutations in genes encoding many of these components have been associated with HSCR in human patients, and the majority of these factors are known to affect multiple cellular processes during development.

Between 2012 and 2013, several reviews described in detail many of the identified molecules that are known to play key roles during ENS development (Bergeron et al., 2013; Bondurand and Sham, 2013; Butler Tjaden and Trainor, 2013; Goldstein et al., 2013; Harrison and Shepherd, 2013; Lake and Heuckeroth, 2013; McKeown et al., 2013; Musser and Southard-Smith, 2013; Obermayr et al., 2013; Young, 2012). For details concerning what we refer to here as “key historical genes”, we encourage readers to go back to these elegant reviews. In order to highlight new data published over the last five years, we provide here an overview of individual genes that cause aganglionosis and their roles in ENS development when known (Table 1). We complement this with a summary of known genes that impact the ENS although aganglionosis is not evident (Table 2) and a summary of environmental factors that influence ENS development (Table 3). Finally we include a summary of known genetic interactions that affect ENS development (Table 4).

The first mouse model of HSCR was generated by targeting the *Ret* gene (Schuchardt et al., 1994). This tyrosine kinase receptor interacts with four distinct ligands [glial cell line-derived neurotrophic factor (Gdnf), neurturin (Nrtn), artemin (Artn) and persephin (Pspn)]. Each of these activates Ret by binding to the glycosylphosphatidylinositol-linked Gdnf family of co-receptors (Gfra1 to 4). In mice, total *Ret* deficiency causes complete intestinal aganglionosis. $Ret^{-/-}$ mice additionally present with kidney agenesis and die at birth. *Gdnf* and *Gfra1* deletions cause nearly identical phenotypes, indicating that they are the critical Ret activators during fetal development (Table 1; (Cacalano et al., 1998; Durbec et al., 1996; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996a; Sanchez et al., 1996)). *Gdnf* haploinsufficiency also leads to severe hypoganglionosis (Table 1; (Gianino et al., 2003; Shen et al., 2002)). In contrast, mutants affecting other Ret ligands or co-receptors present with subtler defects, including reduced nerve fiber density, abnormalities in neurotransmitter release, or hypoganglionosis (Table 2, and see for example (Obermayr et al., 2013; Young, 2012; Zimmer and Puri, 2015)). A large variety of *Ret* mutant mice have been generated over time. These new alleles include mono-isoformic variants as well as serine and tyrosine phosphorylation mutation sites (Table 1). Each of the latter, as well as inactivation of Ret inhibitors, greatly helped decipher downstream signaling pathways involved in ENS ontogenesis (Tables 1 and 2 and references therein). In total, data from these models show that Ret signaling is essential for ENS precursor proliferation, migration, differentiation, survival, and neurite growth. Gdnf/Ret signaling can also influence formation of specific subtypes of neurons, with reduction of neuronal nitric oxide synthase (nNOS) in some mutants (Roberts et al., 2008; Uesaka and Enomoto, 2010). Interestingly, conditional inactivation of *Ret* or *Gfrα1* after gut colonization by ENCCs causes loss of neurons in the colon, suggesting that this signaling pathway is also essential for survival of colonic ENCCs (Uesaka et al., 2007, 2008).

Other cell populations in addition to vagal and sacral neural crest-derived progenitors contribute to formation of enteric ganglia and are Ret signaling dependent. Using genetic fate mapping in mice, Enomoto's group indeed definitively demonstrated that a subset of Schwann cell precursors (SCPs), that invade the gut along extrinsic nerves, adopt a neuronal fate in the postnatal period and contribute to the ENS (Uesaka et al., 2015, 2016). Genetic ablation of Ret in SCPs caused colonic aganglionosis, indicating that SCP-derived neurogenesis is essential for ENS integrity, providing novel insight into the development and disorders of neural crest-derived tissues.

Endothelin-3 (Edn3, a member of the 21 amino acid family of peptides, processed by the Ece1 enzyme) and its seven transmembrane G-coupled receptor (Ednrb) are members of a second pathway shown to play crucial roles in ENS development (Table 1, and references therein). Point mutations in *Edn3* and/or deletion of *Ednrb* are causative for spontaneous mouse mutants called *Lethal spotting*, *Piebald lethal* and *Piebald* respectively (Baynash et al., 1994; Hosoda et al., 1994). The first two mutants, in a manner similar to the genomic knock-out of these genes, present with delay in ENCCs migration within the small intestine, and distal hindgut aganglionosis (Barlow et al., 2003; Baynash et al., 1994; Hosoda et al., 1994). All mutants in the *endo*thelin pathway additionally present with pigmentation defects due to abnormal