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Genetics of enteric neuropathies

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ABSTRACT

Abnormal development or disturbed functioning of the enteric nervous system (ENS), the intrinsic innervation of the gastrointestinal tract, is associated with the development of neuropathic gastrointestinal motility disorders. Here, we review the underlying molecular basis of these disorders and hypothesize that many of them have a common defective biological mechanism. Genetic burden and environmental components affecting this common mechanism are ultimately responsible for disease severity and symptom heterogeneity. We believe that they act together as the fulcrum in a seesaw balanced with harmful and protective factors, and are responsible for a continuum of symptoms ranging from neuronal hyperplasia to absence of neurons.

Contents

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1. Introduction

Normal motility of the gastrointestinal (GI) tract is reliant on complex patterns of smooth muscle contractions and is dependent upon the coordinated action of the enteric nervous system (ENS), smooth muscles cells (SMCs) and interstitial cells of Cajal (ICC). Developmental defects affecting specific cell types or disturbing proper functioning of the ENS, SMCs or ICC, result in variable degrees of abnormal motility, eventually leading to the development of intestinal neuromuscular disorders (Goldstein et al., 2016; Knowles et al., 2013; Panza et al., 2012). Based on the cell type affected, these disorders can be divided into three subtypes: neuropathies (neuronal defects), myopathies (SMC defects), or mesenchymopathies (ICC defects). However, it is important to note that the development and function of these cell types are interconnected (Furness et al., 2014; Gulbransen and Sharkey, 2012; Hao et al., 2016; Sanders et al., 2014), and determining whether a defective cell type is the underlying cause of a disorder, or if the cellular defects are instead a consequence, is not always straightforward. In this review we will focus on enteric neuropathies. We will first describe what is known about the genetics underlying the development of these disorders, and the molecular mechanisms involved in their onset. Moreover, we will discuss enteric neuropathies from a "spectrum" point of view, as many of these disorders are characterized by a continuum of symptoms ranging from severe and evident from birth or even antenatally, to relatively mild or late onset.

2. Enteric neuropathies

Enteric neuropathies can be present along the entire GI tract (see Table 1 for examples). Esophageal achalasia, gastroesophageal reflux disease (GERD), gastroparesis and hypertrophic pyloric stenosis are neurogenic disorders of the upper GI tract. Intestinal Neuronal Dysplasia (IND), the neuronal subtype of chronic intestinal pseudo-obstruction (CIPO), functional constipation, Hirschsprung disease (HSCR) and internal anal sphincter achalasia (IASA) on the other hand, are disorders affecting the lower GI tract. However, it is often common that upper and lower Gl symptoms are present in the same individual. In this section we will focus on each of these disorders and outline what is known about them.

2.1. Esophageal achalasia

Patients with esophageal achalasia are characterized by abnormal esophageal contractility with lack of coordinated peristalsis. In addition, the lower esophageal sphincter (LES) does not relax due to disruption of endogenous innervation (Pandolfino and Gawron, 2015), leading to an elevated resting pressure. Achalasia can result from neuronal damage caused by an autoimmune disorder (Kraichely et al., 2010), or due to specific infections (Becker et al., 2016; Boeckxstaens, 2008; de Oliveira et al., 1995). Histological examinations of patient material revealed severe reductions in myenteric ganglia (Goldblum et al., 1994), nitric oxide synthase producing neurons (nNOS) and numbers of ICC (Gockel et al., 2008).

Evidence for a genetic component in specific subsets of patients exists and comes from rare forms of familial esophageal achalasia (Bosher and Shaw, 1981) and genetic syndromes such as Triple A (Achalasia-addisonianism-alacrimia) syndrome (Tullio-Pelet et al., 2000), infantile-onset achalasia and autism (Shteyer et al., 2015; Taketomi et al., 2005), and Alport syndrome (Leichter et al., 1988). Recently, a genetic susceptibility locus was found in the HLA-DQ region (Gockel et al., 2014), linking immune response to an increased genetic risk. Recessive variants present in the Guanylate Cyclase 1, Soluble, Alpha 3 gene (*GUCY1A3*) (Herve et al., 2014), and in the GDP-Mannose Pyrophosphorylase A gene (*GMPPA*) (Koehler et al., 2013) were also found in patients where achalasia is part of a complex series of symptoms. However, mouse models where the expression of these two genes has been abolished showed no signs of impaired esophageal peristalsis (Buys et al., 2013; Lyon et al., 1996). The only mouse models described to date which showed achalasia-type features are the Nitric Oxide Synthase 1 (*Nos1*) (Sivarao et al., 2001), the Association (RalGDS/AF-6) Domain Family Member 1 (*Rassf1a*) (van der Weyden et al., 2009), the Sprouty RTK Signaling Antagonist 2 (*Spry2*), and the Collagen, Type IV, Alpha *4 (Col4a4)* (Arnold et al., 2011) mice. To date, no genetic variations in *RASSF1A* or *SPRY2* have been described in patients with achalasia, but recessive pathogenic variants in *NOS1* result in infantile-onset achalasia and autism (Shteyer et al., 2015; Taketomi et al., 2005), and variants in *COL4A4* were identified in patients with X-linked dominant Alport syndrome – deafness – nephropathy, which can also develop achalasia (Mochizuki et al., 1994).

2.2. Gastroesophageal reflux disease

In gastroesophageal reflux disease (GERD) there is a retrograde flow of stomach contents to the esophagus predominantly due to transient relaxation of the lower esophageal sphincter (LES), independent of swallowing or peristalsis. GERD is a multifactorial condition, in which the autonomic nervous system, sympathetic (Pfeiffer, 2001) and parasympathetic (Chakraborty et al., 1989; Cunningham et al., 1991; Djeddi et al., 2013), fails to properly control relaxation of the LES. As a consequence, GERD is considered to be an autonomic nervous system defect, not an enteric neuropathy, but patients with GERD can also have motility defects in the upper (Lundell et al., 1996) and lower GI tract, as seen for instance in Cornelia de Lange syndrome (Deardorff et al., 2012; Luzzani et al., 2003). Unsurprisingly GERD has also been associated with neurological conditions such as cerebral palsy, and can present after esophageal repair of esophageal atresia (Kovesi and Rubin, 2004). To date, no gene(s) have been identified as the causative factor of GERD, although association studies have pointed towards the involvement of the 13q14 region (Champaigne et al., 2009; Hu et al., 2004). Moreover, there are human genetic syndromes where patients develop GERD and for which the corresponding animal model has GI motility problems (Kiefer et al., 2003; Kiefer et al., 2008; Spring et al., 2005), confirming the involvement of a genetic component in the development of this disorder.

2.3. Hypertrophic pyloric stenosis

Infantile hypertrophic pyloric stenosis (IHPS), a common pediatric disorder characterized by projectile vomiting, has been suggested to be caused by lack of coordination between the movements of the pyloric sphincter and the contractions of the stomach (Hayes and Goldenberg, 1957). Patients with congenital

Table 1
Examples of intestinal neuropathies.

Neuromuscular disorder	Location	Clinical manifestation	Neuronal, mesenchymal or muscular	Histological findings	Human associated genes[$]
Upper GI-tract					
Esophageal achalasia	Esophagus	Lack of coordinated esophageal peristalsis	Enteric neuropathy?	Reductions in myenteric ganglia, NOS neurons and ICC	Mostly susceptibility loci as a feature in a rare genetic syndrome[a,d]
Gastroesophageal reflux disease	Esophagus	Gastroesophageal reflux	Autonomous nervous system defect	Usually normal[b]	Not yet identified
Gastroparesis	Stomach	Delayed motility and increased sensitivity to mechanical distention	Mesenchymopathy or enteric neuropathy?	Decreased ICC cell numbers are sometimes observed	Susceptibility locus (*CCKAR*); as a feature in a genetic syndrome [a,e]
Infantile pyloric stenosis	Pyloric sphincter	Hypertrophy of pyloric muscle	Myopathy or enteric neuropathy?	Thickened and disorganized longitudinal and circular muscle layers; lack of nerves expressing nNOS and reduced neuronal density	Mostly susceptibility loci; as a feature in a rare genetic syndrome[a,f]
Lower GI-tract					
Chronic Intestinal Pseudo-Obstruction	Small intestine	Bowel obstruction	Different subtypes: Enteric neuropathy; mesenchymopathy and myopathy	Inflammation of the lamina propria and myenteric plexus; reduced ICC number	Depending on subtype e.g. *RAD21*, *SGOL1*, *POLG*, *FLNA*; *SOX10*; *L1CAM*[g]
Intestinal neuronal dysplasia	Large intestine	Bowel obstruction	Myopathy or enteric neuropathy	Giant cells in the submucosal nerve plexus	Not yet identified
Slow transit constipation	Intestine	Constipation	Myopathy or enteric neuropathy	None	Mostly susceptibility loci; as a feature in a genetic syndrome[a,h]
Hirschsprung disease	Colon[c]	Bowel obstruction	Enteric neuropathy	Lack of enteric neurons	Mainly *RET* and *EDNRB*; few susceptibility loci; as a feature in a rare genetic syndrome[a,i]
Internal anal sphincter achalasia	Anus	Obstructed defecation	Enteric neuropathy	None	Not yet identified; as a feature in a rare genetic syndrome[a]

Abbreviations: GI: gastrointestinal tract, ICC: interstitial cells of Cajal, NOS; Nitric oxide synthase.

[$] Genes affected in animal models and human and corroborated reciprocally with an intestinal dysmotility phenotype.
[a] See the OMIM (Amberger et al., 2015) and The London Dysmorphology Database (Winter and Baraitser, 1987).
[b] Sometimes inflammation of the esophageal mucosa occurs as a secondary effect to GERD.
[c] Other segments of the intestine can also be affected in addition to the colon.
[d] Boeckxstaens et al., 2014; Gockel et al., 2014; Wouters et al., 2014.
[e] Rettenbacher and Reubi, 2001; Tahara et al., 2009.
[f] Peeters et al., 2012.
[g] Bonora et al., 2015; Chetaille et al., 2014; Clayton-Smith et al., 2009; Deglincerti et al., 2007; Kapur et al., 2010; Nishigaki et al., 2003; Van Goethem et al., 2003; Verny et al., 2008; Vondrackova et al., 2014.
[h] Peeters et al., 2011.
[i] Alves et al., 2013; Amiel et al., 2008.

hypertrophic pyloric stenosis have decreased innervation of the circular muscle of the pylorus and no nitric oxide synthase activity (Vanderwinden et al., 1992), which has been shown to contribute to a failure of relaxation. Although an involvement of all three cell types, enteric neurons, SMCs, and ICC, has been suggested, a neurological etiology of IHPS is further suspected from syndromic forms, human association studies, and animal models.

To date, the main candidate gene for IHPS is *NOS1,* which was identified in Genome Wide Association studies (GWAS) conducted in a series of pyloric stenosis cases (Chung et al., 1996; Svenningsson et al., 2012). The involvement of this gene in IHPS was further corroborated by the development of the phenotype by the *Nos1* knockout mice (Huang et al., 1993). Moreover, the Glial Cell Derived Neurotrophic Factor gene (*GDNF*) has been associated with this disease, as *Gdnf* homozygous knockout mice showed hypoganglionic intestines, a dilated duodenum and pyloric stenosis (Sanchez et al., 1996). As for other GWAs, there may be population specific effects (Miao et al., 2010).

2.4. Gastroparesis

Gastroparesis is a GI motility disorder characterized by delayed gastric emptying. It can have various causes and is often difficult to distinguish from functional dyspepsia (Stanghellini and Tack, 2014). Histologically, decreased numbers of myenteric neurons as well as ICC (Bernard et al., 2014; Grover et al., 2012) have been found in patients suffering from this disorder. Gastroparesis can occur as an isolated event, but it can also be found as a symptom in Mitochondrial recessive ataxia syndrome caused by variants in the Polymerase (DNA Directed), Gamma gene (*POLG*) (Van Goethem et al., 2004), Mitochondrial DNA depletion syndrome resulting from deleterious genetic variation in the Thymidine Phosphorylase gene (*TYMP*) (Gamez et al., 2002), and in Myotonic dystrophy caused by variants in the Dystrophia Myotonica-Protein Kinase gene (*DMPK*) (Bodensteiner and Grunow, 1984). Association studies have also linked the Cholecystokinin A Receptor gene *(CCKAR)* to the development of gastroparesis (Rettenbacher and Reubi, 2001; Tahara et al., 2009). However, knockout mouse models for *Cckar* and *Cckbr* showed contradictory results. While *Cckar* knockout mice (Miyasaka et al., 2004; Takiguchi et al., 2002) had an increased transit time (Miyasaka et al., 2004; Takiguchi et al., 2002), *Cckbr* knockouts had enhanced gastric emptying (Miyasaka et al., 2004; Nagata et al., 1996). Knockout mouse models for *NOS1* (Huang et al., 1993; Sivarao et al., 2008), the Solute Carrier Family 18 gene (*Slc18a2*) (Chung et al., 1996; Shteyer et al., 2015; Svenningsson et al., 2012; Taylor et al., 2009), and the gene trap ROSA 26 Philippe Soriano [*Gt(ROSA)26S*] (Klein et al., 2013) have all developed gastroparesis. However, no variants in these genes have been identified in patients suffering from this condition.

2.5. Intestinal neuronal dysplasia

Intestinal neuronal dysplasia (IND) is a rare pediatric disorder characterized by bowel obstruction. It is caused by a defect in GI innervation as a result of dysplastic embryonic development of the ENS (Vougas et al., 2014), and can be classified in two subtypes: type A (IND-A) and type B (IND-B). IND-A is the rarest form (5–15%) and is characterized by hypo/aplastic sympathetic innervation. It classically presents with intestinal obstruction in early infant life together with necrotizing enterocolitis (Fadda et al., 1983; Rajalakshmi et al., 2003; Schofield and Yunis, 1992). IND-B is more common (70–95% of cases), and is characterized by abnormal parasympathetic innervation. It can exist as an isolated feature or in association with HSCR (25–35% of the cases), which can explain the recurrence of signs and symptoms following resection of the aganglionic segment (Fadda et al., 1983; Puri, 1997; Rajalakshmi et al., 2003). The diagnosis of IND-B is not always straightforward, as symptoms normally mimic the ones found in HSCR. IND-B is also considered to be a controversial disorder, as there is a high degree of inter-observer variability (Koletzko et al., 1999). However, the presence of giant cells in the submucosal nerve plexus of the colon is a pre-requisite for diagnosis (Meier-Ruge et al., 2006). Due to the fact that healthy infants can show a high number of cells per enteric ganglia, IND-B cannot be diagnosed prior to one year of age.

A genetic component is believed to play a role in the development of IND-A and IND-B based on reported mouse models (Hatano et al., 1997; Shirasawa et al., 1997), but no gene has been yet identified as the causative factor of these diseases. However, expression of the Phosphatase and tensin homolog gene (*PTEN*) was found to be reduced in patients with IND-B, and to be absent in the aganglionic segment of HSCR patients (O'Donnell and Puri, 2011). *Pten* homozygous conditional knockout mice have hyperganglionosis and are reported to develop intestinal pseudo-obstruction (Puig et al., 2009). Therefore, these findings suggest that the PTEN/PI3K/AKT signaling pathway, an important driver of ENS neurogenesis (Becker et al., 2013; Puig et al., 2009), may play a role in the development of IND. Recently, variants on the *ACTG2* gene, encoding gamma 2 enteric actin, a protein crucial for correct enteric muscle contraction, were found in three patients affected by Chronic Intestinal Pseudo Obstruction (CIPO) and with a histological evaluation which fulfilled the diagnostic criteria for IND-B (Matera et al., 2016). Similarly, other histopathological studies of the myenteric and submucosal plexuses reported abnormalities of ganglion cells in CIPO and Megacystis Microcolon Intestinal Hypoperistalsis syndrome patients carrying *ACTG2* variants (Holla et al., 2014; Puri and Shinkai, 2005). These results rule out the histopathological IND picture as a feature peculiar of the sole intestinal innervation defects, suggesting ENS abnormalities can occur either alongside, in parallel or subsequent to a primary non-ENS defect.

2.6. Chronic intestinal pseudo obstruction

Chronic intestinal pseudo-obstruction (CIPO) comprises a variety of conditions characterized by failure of GI motility without apparent signs of lesions explaining the anomaly (Cogliandro et al., 2007). Histological observations categorize CIPO in different classes depending on the involvement of enteric neurons, SMCs and ICC (Cogliandro et al., 2007; De Giorgio et al., 2004). The current understanding of its etiopathogenesis is very limited. Families with several affected members have been described and both syndromic and non-syndromic forms of CIPO have been reported with three types of inheritance patterns proposed: dominant, recessive and X-linked. As a consequence, several genes have been associated with CIPO, but most of these genes have been described for the myopathic type (Gauthier et al., 2014; Kapur et al., 2010; Lehtonen et al., 2012; Matera et al., 2016; Wangler et al., 2014). For the other types of CIPO the exact functional mechanism is not yet known (Bonora et al., 2015; Chetaille et al., 2014; Deglincerti et al., 2007; Nishigaki et al., 2003; Van Goethem et al., 2003; Verny et al., 2008; Vondrackova et al., 2014). However, *de novo* heterozygous variants in the SRY (Sex Determining Region Y)-Box 10 gene (*SOX10*) have been reported in a patient where CIPO was part of a complex group of symptoms associated with Shah-Waardenburg syndrome (Pingault et al., 2002). Variants in *SOX10* have also been found in HSCR patients (Pingault et al., 1998).

2.7. Functional constipation

Functional constipation is a common problem in the pediatric population and is characterized by infrequent hard and painful

defecation often accompanied by involuntary loss of stool (Benninga et al., 2016; Burgers et al., 2012; Hyams et al., 2016; Tabbers et al., 2014). A proportion of affected children appear to suffer from more severe forms of constipation, including slow transit constipation (STC). Severe childhood constipation can sometimes be difficult to distinguish from CIPO, as both conditions may represent a spectrum of motility problems. However, CIPO by definition involves the small intestine, patients have episodes of intestinal obstruction with vomiting, often bilious, and on the whole their motility problems are more severe. In a proportion of patients with more severe forms of childhood constipation reduction in ICC numbers has been suggested to be a contributing factor (He et al., 2000; Lyford et al., 2002; van den Berg et al., 2009), but this is not conclusive (Knowles and Farrugia, 2011), Abnormal levels of neuropeptides such as vasoactive intestinal peptide (Koch et al., 1988), serotonin (Zhao et al., 2002) and substance P (Tzavella et al., 1996) have also been detected in patients with severe constipation, especially in STC (King et al., 2006, 2010; Stanton et al., 2003; Yik et al., 2011).

In children affected with functional constipation there often appears to be a positive history of constipation in the family (Osatakul and Puetpaiboon, 2014). However, knowledge of the genetic factors contributing to the disease is limited. Genetic associations with the Tachykinin Receptors 1 (*TACR1*) and 3 (*TACR3*), V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (*KIT*) and *NOS1* genes have been identified in a candidate gene association study (Garcia-Barcelo et al., 2007). Several other direct sequencing studies investigated the presence of variants in candidate genes such as Neurturin (*NRTN*) (Chen et al., 2002; Heuckeroth et al., 1999), REarraged during Transfection (*RET*) (Knowles et al., 2000) and *KIT* (Tong et al., 2006), or evaluated chromosomal anomalies (Rossi et al., 2007) in patients with intractable constipation. Childhood constipation also presents as a symptom in several genetic syndromes (Peeters et al., 2011).

2.8. Hirschsprung disease

Hirschsprung disease (HSCR) is the most intensively investigated enteric neuropathy. It occurs due to abnormal development of the ENS, which results in aganglionosis of the distal GI tract (McKeown et al., 2013; Obermayr et al., 2013). The length over which the aganglionosis extends can vary and HSCR is classified as short segment, long segment, total colonic and total intestinal aganglionosis. HSCR is considered to be a highly heritable disorder with both dominant and recessive modes of inheritance described. Although familial cases have been reported, most cases are sporadic and are considered to be polygenic or multifactorial in origin. The first gene to be associated with HSCR was *RET*, a proto-oncogene located on chromosome 10. The involvement of this gene in HSCR was found by linkage analysis in large multigenerational families (Angrist et al., 1993; Lyonnet et al., 1993), and to date, the presence of *RET* variants is still considered to be the major contributing factor for HSCR (Edery et al., 1994a; Romeo et al., 1994). Deleterious variants in *RET* are present in 50% of familial and 15–20% of sporadic HSCR patients (Amiel et al., 2008). The severity of the mutation, its dosage and the presence of modifying factors are believed to influence the length of the aganglionic segment (Basel-Vanagaite et al., 2007; Edery et al., 1994b). Most of these variants are rare and have a high penetrance. However, a few very common low penetrant variants have also been identified in HSCR patients via locus specificity and GWAS (Garcia-Barcelo et al., 2009; Jiang et al., 2015; Kim et al., 2014). Unsurprisingly, the strongest association was found in a haplotype covering part of the *RET* locus (Burzynski et al., 2005; Emison et al., 2005). On this haplotype, several variants within intron 1 of *RET* were proven to disturb the function of a *RET* enhancer and were believed to contribute to disease development (Emison et al., 2010; Sribudiani et al., 2011). These non-coding single nucleotide polymorphisms (SNPs) are found in approximately 60–80% of all HSCR patients, and in 50% of them the haplotype is present in a homozygous state, increasing the risk for HSCR 10–20 fold (Emison et al., 2005; Burzynski et al., 2005).

The Endothelin Receptor type B gene (*EDNRB*) was the second gene to be associated with HSCR. Interestingly, *EDNRB* was identified in a large Mennonite kindred in which HSCR was found in association with Waardenburg Syndrome (Puffenberger et al., 1994a, 1994b). *EDNRB* has also been shown to epistatically interact with *RET* (Carrasquillo et al., 2002; McCallion et al., 2003). After these initial findings, several other genes have been found to play a role in HSCR, and to date more than 16 genes have been identified, most of which are not fully penetrant (Alves et al., 2013). As for *EDNRB,* almost all these genes have been shown to cause rare syndromic forms of HSCR. Moreover, it is believed that epistatic interactions play a major role in HSCR development, making this disease a paradigm for other heterogeneous or oligogenic disorders (de Pontual et al., 2007, 2009).

2.9. Internal anal sphincter achalasia

In patients with Internal anal sphincter achalasia (IASA) ganglion cells are present in rectal biopsies. However, the internal anal sphincter fails to relax, resulting in a Hirschsprung like phenotype (Ciamarra et al., 2003; De Caluwe et al., 2001; Doodnath and Puri, 2009; Neilson and Yazbeck, 1990). Different developmental defects have been proposed to explain IASA onset: nitrergic nerve depletion (Hirakawa et al., 1995), altered distributions of ICC (Piotrowska et al., 2003), and defective neuronal innervation of the neuromuscular junction (Oue and Puri, 1999). Moreover, an underlying genetic cause is suspected in at least some of these patients, as IASA can segregate within families (Celik et al., 1995; de la Portilla et al., 2005; Kamm et al., 1991) and is found as a symptom in X-linked alpha thalassemia mental retardation (ATR-X) syndrome (Martucciello et al., 2006). However, no genetic factors have been identified to date.

3. Common denominators in GI enteric neuropathies

For most GI neuropathies the precise etiology is as yet unknown (see above). However, a deficit in neuronal composition and/or neuronal numbers seems to underlay the abnormal GI motility characteristic of these disorders. In this section we present evidence that most of the GI neuropathies share a cellular and/or genetic basis. Moreover, we propose a seesaw model for neuronal motility to explain differences in disease severity.

3.1. Neuronal composition

Normal neuronal composition of the GI tract is crucial for intestinal peristalsis, as absence or altered ratios of specific neuronal subtypes are known to lead to abnormal GI motility. Although defects in neuronal composition can result from anomalies in transcriptional programs responsible for neuronal subtype specification, knowledge regarding development of neuronal subtypes, axonal guidance or neuronal control of motility is limited (Sasselli et al., 2012). What we do know is that NOS neurons are the only neuronal subtype implicated in several intestinal neuromuscular disorders of the GI tract, such as HSCR, gastroparesis, hypertrophic pyloric stenosis and esophageal achalasia (Chung et al., 1996; Huang et al., 1993; Shteyer et al., 2015; Sivarao et al., 2008; Svenningsson et al., 2012; Taketomi et al., 2005). Serotonergic neurons, as well as intrinsic primary afferent neurons (IPANs) and

substance P positive neurons, are also highly important for proper peristalsis (Gershon, 2013). Therefore, it is tempting to suggest that defects in genes or networks regulating the differentiation of neuronal subtypes or functional establishment of neuronal circuits, may be part of the etiopathology of enteric neuropathies. In this context, it is worth mentioning that loss of function variants in the Achaete-Scute Family BHLH Transcription Factor 1 gene (*ASCL1*, previously *MASH1*) result in loss of specific MASH1-dependent enteric neuronal precursors such as serotonergic and NOS neurons, in the Ret/GFRa1/GDNF signaling-independent part of the esophagus and stomach in mice (Blaugrund et al., 1996; Gershon and Ratcliffe, 2004). Mice deficient for the tryptophan hydroxylase 2 enzyme involved in serotonin biosynthesis have reduced neuronal density and increased intestinal transit times (Li et al., 2011), and knockout mice for the Tyrosine Kinase Receptor C (*TrkC*), the receptor for Neurotrophin-3, have fewer submucosal IPAN neurons (Chalazonitis et al., 2001). Moreover, there are less substance P positive neurons in some children with slow transit constipation and in multiple endocrine neoplasia type 2B (MEN2B), a condition caused by activating *RET* mutations (Hutson et al., 1996; King et al., 2006). The same could also apply for the numbers of ICC or combinations of ICC and neurons. For instance, ICC numbers are reduced in pyloric stenosis (Vanderwinden et al., 1996a) and gastroparesis, and ICC networks are also disrupted in HSCR (Vanderwinden et al., 1996b). However, pyloric stenosis and intestinal aganglionosis are not frequently seen together. All things considered, there seems to be increasing evidence for the importance of maintaining the balance of different neuronal subtypes for enteric motility to occur normally.

3.2. Neuronal numbers

As mentioned previously, HSCR and many other GI neuropathies are characterized by a reduction in neuronal numbers. This fact lead us to hypothesize that a substantial proportion of these neuropathies may well have a common genetic etiology. Therefore, we postulate that the lack of neurons found in HSCR is part of a spectrum of ENS related diseases of which HSCR is at one end and hyperganglionosis is at the other. As *RET* is the main gene involved in HSCR, it is tempting to say that RET is the crucial factor for the establishment of proper numbers of neurons (ganglia), and variation in RET expression levels or protein function may be responsible for a continuum of disorders ranging from hypoganglionosis to hyperganglionosis. Several lines of evidence corroborate this hypothesis. Pathogenic *RET* variants can be inherited from parents without HSCR; the aganglionic segment is variable in length in patients carrying these variants, ranging from short segment HSCR to total intestinal aganglionosis, and pseudo-obstruction to hypoganglionosis and slow peristalsis (Lui et al., 2008); and *RET* activating variants are found in MEN2B (Hofstra et al., 1994), a disorder where patients show signs of hyperganglionosis in addition to several other malignancies. In light of this evidence, we believe that RET could be a common denominator for GI neuropathies characterized by a reduction in neuronal number.

3.3. Seesaw model of motility disorder development

Using HSCR as an example, we propose a model for disturbed ENS development in which harmful and protective factors balance on a fulcrum representing a disease-specific genetic predisposition (see Fig. 1). The balance of this seesaw can be disturbed by genetic burden as well as non-genetic factors such as epigenetics and environmental exposures.

Epigenetic changes have been linked to the development and progression of several disorders, including HSCR. Recently,

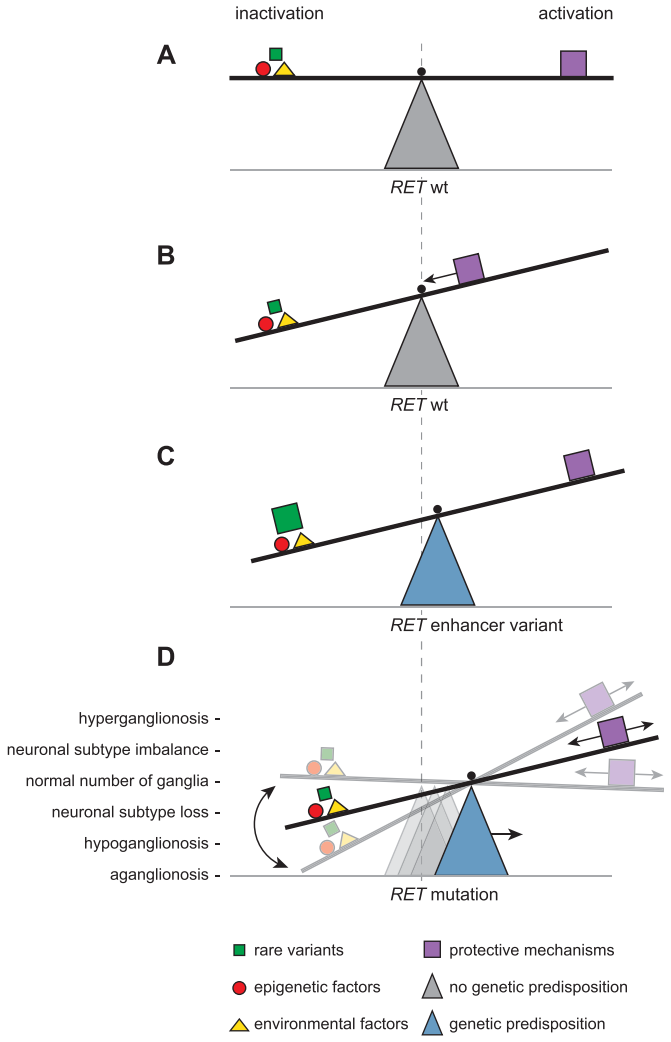

Fig. 1. Seesaw of GI motility disorder development using HSCR as an example. Depending on the genetic background, harmful and protective factors may shift the fulcrum and lead to a "disease spectrum balancing seesaw", in which the weight of specific factors and movement of the fulcrum reflects a spectrum of GI motility disorders ranging from hyperganglionosis to normal development, and finally to aganglionosis. (A) Seesaw, with fulcrum represented by wildtype *RET*. There is a balance between harmful and protective factors. (B) When the contributions of protective factors decrease, harmful factors tilt the balance to abnormal development. This might also happen if: (C) the burden of rare variants in other genes increases or if the *RET* intron 1 variant shifts the fulcrum of the seesaw changing the balancing point; (D) a deleterious *RET* variant has a higher penetrance shifting the fulcrum with increasing deleteriousness of the variant.

variants in the gene encoding for the *de novo* methyltransferase 3B (*DNMT3B*) have been found to lead to decreased expression of this gene in enteric precursors isolated from HSCR patients (Torroglosa et al., 2014). Moreover, there has been increasing evidence that the expression of several known HSCR genes, such as *RET* and *EDNRB*, can be controlled by DNA methylation of their promoter regions. For more details about the involvement of epigenetics in HSCR, please see the review of Torroglosa et al published in this issue of the journal.

Environmental factors are also known to influence ENS development (Heuckeroth and Schafer, 2016) and have been shown to play a role in enteric neuropathies. For HSCR development a relationship with maternal hyperthermia was previously suggested (Lipson, 1988), although this has been disputed by others (Larsson et al., 1989). Vitamin A levels have also been shown to impact HSCR penetrance and severity. Depletion of this vitamin causes distal bowel aganglionosis in mice, as correct intake levels of

vitamin A seem to be crucial for proper migration and polarization of ENS precursor cells and lamellipodia formation in mice (Fu et al., 2010). Moreover, the use of mycophenolic acid and ibuprofen, commonly used as immunosuppressant and anti-inflammatory drugs respectively, have been associated with HSCR in two other studies. Their application during development of zebrafish, chick and mouse animal models led to reduced ENS precursor migration as well as defective lamellipodia formation, cell proliferation, and survival (Lake et al., 2013; Schill et al., 2016). However, it is worth mentioning that in these three studies the genetic background of the animals used played a crucial role in disease onset and severity, suggesting that environmental factors may shift the balance from normal to abnormal ENS development in an already genetically compromised individual by the presence of, for instance, a variant in *RET*.

4. Conclusions

GI motility is regulated by a large number of neuronal and muscular processes. These processes need to be tightly regulated, as they are known to influence each other. Here we postulate that many of the GI-related enteric neuropathies have a common basis, as most of them are characterized by a deficit in neuronal composition and/or neuronal numbers. Based on this assumption, we propose a seesaw model for development of enteric neuropathies in which genetic and non-genetics factors contribute to disease onset and severity. We believe that in this model, the fulcrum is a key component of the underlying biological defect. Although we have no strong evidence for this hypothesis as yet, we do see overlap between diseases and genes involved. Possibly, GI neuropathies characterized by quantitative differences in neuronal subtypes might form a group of disorders in which NOS1 is the key component, as it is required for circular muscle and sphincter relaxation in esophageal achalasia, hypertrophic pyloric stenosis and IASA (Rivera et al., 2011). For disorders with numerical loss of neurons RET could be the common denominator, since it is important for survival, proliferation and migration of enteric neuronal precursor cells (Natarajan et al., 2002; Uesaka and Enomoto, 2010; Uesaka et al., 2008). RET could also be the fulcrum in neuropathies involving specific neuronal subtypes, as RET levels or RET ligands are important for neuronal differentiation, neurite outgrowth and axon patterning (Buj-Bello et al., 1997; Fleming et al., 2015; Heuckeroth et al., 1999; Rossi et al., 1999; Uesaka et al., 2013). Whether this model is true- and underlies the etiology of patients in which the genetic causes are unknown remains elusive. This is partly due to the fact that subtle differences in neuronal numbers are hard, if not impossible, to determine using current histopathological approaches. Nevertheless, to prove this hypothesis and better understand disease pathophysiology we need to examine the GI tract of enteric neuropathy patients more thoroughly, perform rigorous pathological examination of GI biopsies (Swaminathan et al., 2015), and combine these observations with whole exome or genome sequencing results.

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