Chapter 13
Postnatal Development of the Mouse Enteric Nervous System

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Owing to over three decades of research, we now have a good understanding of the genetic and molecular control of enteric nervous system (ENS) development during embryonic and prenatal stages. On the other hand, it has only just become clear that a substantial process of ENS maturation occurs after birth (Hao et al. 2013a). During postnatal stages, in addition to genetic influences, ENS development is also potentially affected by the external environment. Thus it is possible that manipulating certain environmental factors could help prevent or reduce motility disorders. However the genetic and environmental factors that regulate postnatal ENS development remain unknown. Researchers have used a variety of animal models that are easy to manipulate genetically or experimentally, and have short gestational periods, to understand the development of the ENS. Notably, due to the availability of mouse models for several human enteric neuropathies, many studies have used the mature and developing murine ENS as a model. Here, I will discuss recent advances in knowledge about postnatal development of the murine ENS, and highlight future directions for this emerging research field.

Development of Enteric Ganglionated Plexuses

The ENS is a network of neurons and glia residing within two major ganglionated plexuses embedded along the wall of the gastrointestinal tract (Furness 2012). The submucous plexus lies between the mucosa and circular muscle, the myenteric plexus is sandwiched between the longitudinal and circular muscles.

The ENS derives mainly from the vagal neural crest. Similar to other parts of the nervous system, development of the ENS comprises of a series of overlapping

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stages: migration, proliferation, differentiation and formation of synapses (Hao et al. 2013a; Sasselli et al. 2012). While the formation of the myenteric plexus precedes the submucous plexus in mice (Jiang et al. 2003; Uesaka et al. 2013), much is still unknown about how development of neurons in one plexus influences the other. The mature and developing myenteric plexus has been well studied compared to the submucous plexus.

Maturation of Cholinergic and Nitrergic Neurons

In the mature ENS, cholinergic (express the enzyme choline acetyltransferase, ChAT) and nitrergic (express the enzyme neuronal nitric oxide synthase, nNOS) neurons are typically characterized as separate neuronal populations (Foong et al. 2014; Sang and Young 1998). Cholinergic neurons are excitatory neurons and function as motor neurons, interneurons and sensory neurons, while nitrergic neurons can be inhibitory motor neurons or interneurons.

Significant numbers of myenteric neurons are still being born (exiting the cell cycle) between P0 and P10 in mice (Bergner et al. 2014; Laranjeira et al. 2011; Pham et al. 1991). Submucosal neurons tend to exit the cell cycle and differentiate later than myenteric neurons (McKeown et al. 2001; Pham et al. 1991). Some neuronal precursors transiently express certain neurotransmitter synthetic enzymes during fetal or early postnatal development (Baetge and Gershon 1989; Bergner et al. 2014; Gershon et al. 1993; Hao et al. 2013b; Obermayr et al. 2013). For example, in the small intestine, some submucosal and myenteric neurons that do not express nNOS in the mature gut transiently express nNOS during embryonic and early postnatal development (Bergner et al. 2014; Young and Ciampoli 1998). Nonetheless, segregation between nitrergic and cholinergic neurons seems to be established early in development (Hao et al. 2013b).

The proportion of nNOS myenteric neurons in the small intestine does not change significantly postnatally, whereas nNOS submucosal neurons decline from 50 % at P0 to only 3 % in adult mice (Young and Ciampoli 1998). Research into the development of ChAT neurons has been limited by technical difficulties until very recently. Uptake of 3[H]-choline at E10–E12 suggested that ChAT+ cells appear very early in development (Rothman and Gershon 1982). However, several ChAT antisera failed to detect any neurons in the gut until late embryonic stages. To overcome this problem, two recent studies used mouse fluorescent reporter lines to detect ChAT (Hao et al. 2013b; Erickson et al. 2014). The first used Cre-lox technology (*ChAT-Cre* mice in combination with a fluorescent reporter line) and detected the first ChAT+ neurons in the gut at E11.5. In this *ChAT-Cre:R26R* line, cholinergic neurons gradually increase in number during late embryonic and postnatal stages, and at birth, the proportion of cholinergic neurons at birth is still less than half of that in the adult small intestine (Hao et al. 2013b). The second study extended these findings and found that in *ChAT-GFP* mice GFP+ neurons are already present at E10.5; this study was also able to detect ChAT immunoreactive neurons in the gut

E10.5 (Erickson et al. 2014). Furthermore, the proportion of cholinergic neurons in the small intestine was found to achieve adult levels as early as E13.5 (Erickson et al. 2014).

Little is known about the mechanisms regulating neuronal differentiation postnatally, although the role of two transcription factors in postnatal neuronal differentiation has been examined. *Hand2* haploinsufficiency results in a reduction of nNOS neurons in embryos, and this reduction persists through to postnatal weeks 6–8 (D'Autreaux et al. 2011). Homeodomain interacting protein kinase 2 (*HIPK2*) is a transcriptional cofactor of bone morphogenetic proteins. Loss of *HIPK2* did not affect enteric neurons during embryogenesis, but caused a progressive decrease in the number of myenteric and submucosal neurons in the caudal gut with increasing postnatal age (Chalazonitis et al. 2011). Thus *HIPK2* may be important for survival and differentiation of enteric neurons postnatally (Chalazonitis et al. 2011). Although not specifically studied, many other transcription factors, including *Ascl1* (formally known as *Mash1*) (Okamura and Saga 2008), implicated in prenatal neuronal differentiation could also be involved in the postnatal development of enteric neurons.

Some external factors have been reported to affect enteric neuron differentiation postnatally. For example, the colonization of the intestine by microbiota has been shown to affect ENS postnatal development. The jejunum and ileum of germ-free mice have decreased myenteric nerve density and ganglion size, but an increase in proportion of myenteric nitrergic neurons (Collins et al. 2014). However, whether this is a direct effect of a lack of microbiota remains to be elucidated, as there are other defects in germ-free mice and the ENS defects might be indirect (Yi and Li 2012). Diet has also been shown to affect the postnatal development of the ENS; mice fed on a high fat diet for 8 weeks have decreased numbers of myenteric neurons in the small and large intestine, but submucosal neurons were unaffected (Stenkamp-Strahm et al. 2013; Voss et al. 2013). Notably in the duodenum, nitrergic neurons were particularly affected, while cholinergic neurons were spared (Stenkamp-Strahm et al. 2013). In contrast, a high fat diet was reported to be neuroprotective in the stomach, preventing age-associated loss of nitrergic myenteric neurons (Baudry et al. 2012).

Maturation of Neuronal Morphology

As in other species, adult enteric neurons in the mouse have either a single axon or multiple axons (Dogiel type II, DII) (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008). Most myenteric uniaxonal neurons only have lamellar dendrites, while DII neurons have smooth cell bodies and typically project their axons circumferentially (Foong et al. 2012; Nurgali et al. 2004).

The first enteric neurons to differentiate have single long, anally-projecting axon-like processes (Hao et al. 2013a; Young et al. 2002, 2014). Orally and circumferentially-projecting neurons appear later. Unlike adults, most uniaxonal

embryonic and early postnatal myenteric neurons have multiple filamentous dendrite-like structures instead of lamellar dendrites (Foong et al. 2012). Maturation of dendritic morphology and axonal projection lengths of uniaxonal neurons persists in postnatal stages, even after P10. DII neurons are present by birth and their long processes project nearly around the entire gut circumference at P10. Despite significant growth in gut circumference between P10 and adult, the lengths of DII neuron axons do not increase after P10 (Foong et al. 2012). The morphological changes in dendrites and axons after birth suggests that synapse formation remains dynamic postnatally; raising the possibility that ENS connectivity is vulnerable to environmental factors.

Maturation of Electrophysiological Properties

Enteric neurons are usually characterized into two classes. One group fires action potentials with short repolarizations (S-type), while the other group of neurons fires action potentials with long after-hyperpolarization potentials (AH-type). S-type myenteric neurons are typically uniaxonal while AH-type neurons exhibit DII morphology (Foong et al. 2012; Nurgali et al. 2004).

The electrophysiological properties of early postnatal myenteric neurons have been examined only very recently. At birth, the two adult-like classes of myenteric neurons are present, with distinctive electrophysiological and corresponding morphological properties, but these properties are not yet mature (Foong et al. 2012). Furthermore, the two classes of neurons develop asynchronously. S-type neurons seem to mature electrophysiologically first. A prominent Ca^{2+}-mediated afterdepolarizing potential is observed in DII neurons at P0 and P10 that is significantly larger than that recorded in their adult counterparts (Foong et al. 2012). In other parts of the nervous system, neuronal activity and increased intracellular calcium concentration affect development (Young et al. 2014), however, the mechanisms regulating the postnatal maturation of enteric neurons remain to be identified.

Maturation of Synaptic Profile

Adult enteric neurons receive a variety of synaptic inputs including fast and slow excitatory postsynaptic potentials (EPSPs), and inhibitory postsynaptic potentials (IPSPs) (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008). The vast majority of enteric neurons exhibit fast EPSPs and only the neurotransmitters mediating fast transmission have been investigated in mice to date. Acetylcholine activating nicotinic receptors is the main mode of fast transmission, but ATP acting via P2X receptors is also involved (Foong et al. 2012, 2014; Nurgali et al. 2004; Wong et al. 2008).

At P0, S-type neurons exhibit fast EPSPs, and by P10, like adults, these fast EPSPs are mediated by nicotinic receptors (Foong et al. 2012). At P10-11, there is still few, if any, slow EPSPS or IPSPs (Foong et al. 2012). Thus, in combination with the significant growth of gut size, maturation of dendritic structure and axonal projection that occurs from P10 to adulthood, it is evident that the formation and maturation of synapses occurs over a protracted period of time.

Little is known about the regulation of synaptogenesis in the ENS. However, loss of *Hipk2* and the absence of gut microbiota reduced intraganglionic synapses and nerve density respectively in postnatal myenteric plexus (Chalazonitis et al. 2011; Collins et al. 2014).

Maturation of Neurally-Mediated Motility Patterns

Motility in the mature intestine involves interactions between enteric neurons, interstitial cells of Cajal and intrinsic smooth muscle (Huizinga and Lammers 2009). Motility patterns differ in different regions of the gut, but mainly involves a combination of mixing behaviours to facilitate digestion and absorption of nutrients, and propulsion, to push the gut contents along the GIT (Bornstein et al. 2004; Burns et al. 2009; Costa et al. 2013; Huizinga and Lammers 2009).

It is essential for the duodenum to be functional by birth in order for the newborn to digest and absorb milk nutrients. The first propagating motility patterns detected in the gut are non-neuronal. In the duodenum, neurally-mediated motility commences just prior to birth, at E18.5 (Roberts et al. 2010). Conversely, neurally-mediated motility in the colon commences several days after birth. By P6, the neural circuit underlying colonic migrating motor complexes (CMMCs) is present; however, CMMCs are only induced by blocking NOS (Roberts et al. 2007). In E18.5 and P0 duodenum, inhibition of nNOS induced or increases the frequency of contraction complexes (Roberts et al. 2010). In addition, during development the circular muscle appears to receive innervation from nNOS+ fibres prior to ChAT+ fibres. The number and density of ChAT+ fibres increase significantly after birth (Hao et al. 2013b; Roberts et al. 2007). Thus overall these pharmacological and immunohistochemical studies suggest that smooth muscle cells are tonically inhibited by NO until sufficient ChAT+ fibres are present to induce contractions. However, a re-examination of the number and density of ChAT+ fibres during development is now necessary since ChAT+ neurons are recently reported to achieve adult proportions by E13 in the small intestine and by P0 in the proximal colon (Erickson et al. 2014). In both duodenum and colon, motility patterns seem to be mature by P10 (Hao et al. 2013b; Roberts et al. 2007, 2010).

Some genetic and environmental factors that affect postnatal development of neurons also elicit profound effects on gut motility. Indeed, abnormal numbers of neuronal subtypes and nerve density due to impaired trophic factor signalling, absence of gut microbiota and prolonged ingestion of high fat diets all led to altered gut motility (Baudry et al. 2012; Chalazonitis et al. 2011; Collins et al. 2014; Mushref and Srinivasan 2013).

Postnatal Gut and Therapy

Understanding how the ENS develops postnatally could assist in developing therapies for gut motility disorders. Defects in ENS development result in pediatric motility disorders including Hirschprung disease, a congenital disorder affecting 1:4000–5000 newborns, in which enteric neurons are absent from the distal gut. These patients suffer from severe constipation and require surgery. Current therapies for Hirschprung patients and other gut motility disorders are life-saving, but remain inadequate in addressing other complications including dysmotility and incontinence that can last for years (Hotta et al. 2009).

A potential treatment plan would be to restore the ENS at the aganglionic region of Hirschprung patients. In recent years, the idea of transplanting progenitor cells as therapy for gut motility disorders has generated a lot of interest (Burns and Thapar 2014). Diagnosis of enteric neuropathies typically occurs after birth, thus cell therapy has to be administered postnatally. In fact, it is ideal for progenitor cells to be obtained from a "healthy" gut region of the patients themselves. Neural progenitors can be obtained the postnatal mouse and human gut and that their developmental potential appear to be similar to those obtained from fetal gut (Bondurand et al. 2003; Hotta et al. 2013; Metzger et al. 2009). Furthermore, it has recently been shown that after transplantation into the colon of postnatal mice, ENS progenitors isolated from postnatal gut successfully developed into neurons with the appropriate enteric neuronal subtype properties, including electrophysiological properties (Hotta et al. 2013). While this shows that cell therapy is a promising strategy, there are still significant obstacles to be overcome. In particular, these progenitors need to be able to generate a functional ENS over the patient's aganglionic region (average length 7–10 cm) (Burns and Thapar 2014).

External factors such as diet and gut microbiota found to affect postnatal development could potentially be manipulated to prevent or help rescue the defected ENS. This strategy can be applied on its own or coupled with cell therapies to improve treatment of enteric neuropathies.

Conclusions

Substantial development of enteric neuronal properties continues postnatally. During this time the ENS is vulnerable to factors present in the extra uterine environment. Interaction between the environment and the yet to be identified intrinsic factors regulating ENS maturation will be a fertile source of research in the future.

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